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Ultraviolet Microscopy of X-Irradiated Intestine [1,2]

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Summary

The color-translating ultraviolet microscope was used to study radiation effects in the mouse intestine. Generative cells showed pronounced changes in light absorption at 263 mμ; the light-absorbing clumps normally present in the nucleus disappeared. Later, diffuse, strong absorption was seen in cytoplasm and nucleus; still later, there was very little absorption. The earlier changes occurred uniformly in all generative cells and thus cannot be related to a stage in the mitotic cycle; the late changes were independent of resumption of mitosis. The principal cells had similar but much less pronounced changes in 263 mμ absorption. Later, changes occurred that were related to abnormal development. The Paneth cells showed similar changes in 263 mμ absorption. In addition, they showed early degeneration of a specific (secretory) cytoplasmic structure, followed by speedy recovery.—**J. Nat. Cancer Inst. 21: 813–823, 1958.**

The salient feature of this investigation is the use of the color-translating ultraviolet microscope. The instrument was used, in a rather empirical fashion, to study a process which was reasonably familiar to us. In spite of a limited amount of material and a limited experience, interesting observations resulted.

All of our findings could have been made without this particular instrument. They could be reproduced with a plain ultraviolet microscope and the usual method of evaluation, or even with a conventional light microscope and various cytochemical techniques. The fact is, however, that these observations were not made before we used the color-translating ultraviolet microscope. It is our impression that it is difficult to match the over-all amount of information of various kinds that can be derived from watching the color-translated pictures. The instrument in its present form is difficult to handle and its performance is definitely short of perfect;

[1] Received for publication May 14, 1957.
[2] This work was performed under the auspices of the U. S. Atomic Energy Commission.

accordingly, our findings were obtained through considerable effort. It is hoped that future improvements of the instrument will make its advantages more readily accessible.

The biological process studied was the radiation response of the intestines in mice. The epithelium that lines the small intestine is a renewal tissue (*1*, *2*). Like many renewal tissues, it is highly radiosensitive. Warren and Whipple found, in 1922, that the X-irradiated small intestine is the site of severe and potentially lethal changes (*3*). The evolution of the intestinal pathology following irradiation depends critically on interference with cell production. The generative cells are highly radiosensitive; they disappear after doses of 1 kilorad or more. In the absence of renewal tissue, death occurs after continued loss of cells has caused complete denudation of the gut wall (*4*). Smaller doses result in changes in the generative cells, which are at least partially reversible; their duration and severity depends on the dose. Tillotson and Warren (*5*) have characterized 4 stages in the temporary alteration of the generative cells: 1) there seems to be loss of nucleic acid by degradation and inhibition of synthesis, and mitoses are absent; 2) there is evidence of intensive but largely unsuccessful attempts to synthesize nucleic acid, and mitoses are scant; 3) the cells contain more nucleic acid than normal, and mitoses are abundant; 4) normal conditions are re-established.

Although certain broad outlines of the nature of the acute radiation response of the intestine have been established, much remains to be learned. Therefore, we have investigated the irradiated intestine with the help of a color-translating ultraviolet microscope. We were not the first to do this. Several investigators have used ultraviolet microscopes on the irradiated intestines, and Tillotson and Warren (*5*) worked with an instrument very similar to the one we used. Their studies overlap ours, and our results agree with theirs as far as the overlap goes.

Since we used only small samples because of the large amount of labor involved and since we were not familiar with the results of observation with the color-translating microscope, we have recorded only those pathological changes which were conspicuous and constant.

Materials and Methods

The animals used were 31 young adult female CF #1 mice. They were kept under standard laboratory conditions. Five mice were retained as controls and most of the remainder were given 2000 r or 600 r of X radiation to the whole body. The radiation factors employed were: 250 kvp, filter 0.5 mm. Cu plus 3 mm. Bakelite (HVL 1.5 mm. Cu), dosage rate 245 to 260 r per minute.

The two dosage levels were selected on the basis of previous studies of dose-response relations. A dose of 2000 r is safely beyond the threshold for acute intestinal radiation death; that is, it can be counted on to block cell production long enough to cause denudation of the intestine and subsequent death. A dose of 600 r is known to block production of cells for approximately 18 hours; this is followed by a recovery process as described

by Tillotson and Warren (*5*). The differentiated cells formed are known to be functionally defective for quite some time after irradiation (*4*).

Mice were sacrificed by ether anesthesia at 10 and 30 minutes, 1, 3, 4, and 6 hours, and 1, 2, and 3 days after irradiation. A portion of the jejunum about midway between pylorus and ileocecal valve was excised, cut at 3μ, with the frozen-section method developed by Adamstone and Taylor (*6*), and immediately fixed in 10 percent formalin. This method produces a minimum of artifacts. Unstained sections were mounted in glycerol on Vycor slides for the ultraviolet examination; for comparison, hematoxylin-eosin-stained sections were examined with the conventional microscope.

The color-translating ultraviolet microscope used is 1 of 6 manufactured by the Scientific Specialties Corporation. Except for some modifications, it is similar to the prototype instrument developed at the Polaroid Corporation (*7*, *8*).

The wavelengths used were 263, 240, and 350 mμ. The first was selected because it is known to be strongly absorbed by certain constituents of nucleic acid. The other two were selected empirically as those which seemed to give the most additional information. However, sections photographed at 240 mμ do not markedly differ from those photographed at 263 mμ, except for some details that will be discussed later; 350 mμ gives mostly background color with little contrast.

On each specimen, we photographed 3 different regions extending from the base of the crypt to the tip of the villus; this took 3 to 6 overlapping fields. The slides were studied from a general point of view, without predetermined reference to specific questions. Results obtained with the color-translating ultraviolet microscope are dependent at certain stages on photographic materials and processes. Adequate process control measures were taken. Related details have been discussed elsewhere (*5*, *9*, *10*). In addition, animals were irradiated and sacrificed on such a schedule that similar materials were available at different periods of operation of the color-translating ultraviolet microscope. This gave overlaps in the collection series which served as a check on the operation of the machine and on the reproducibility of the observations. Observations were made from the color-translated images in the microscope and in the auxiliary projector, from black-and-white images in a microfilm reader, and from photographic prints produced under controlled conditions.

Observations and Discussion

Generative Cells: Changes in Amount and Distribution of 263-mμ-Absorbing Material

All descriptions are given in terms of absorption of 263 mμ ultraviolet.

In 4 *controls* (fig. 1) the 263 mμ picture shows little diffuse absorbing material in the cytoplasm and only slightly more in the nucleus. In addition, each nucleus contains several ultraviolet-light-absorbing small clumps of irregular shape. The spaces between cells are dark, which could be due to absorption or scattering.

The fifth control showed diffuse leukocyte infiltration, distortions in the architecture of the Paneth cells, and strong diffuse absorption in the cytoplasm of the generative cell. This was undoubtedly a pathological intestine.

Animals irradiated with 2000 r.—After 1 hour both cytoplasm and nuclei absorb ultraviolet light somewhat more intensely, and the contrast between nucleus and cytoplasm is lessened (2 animals, fig. 2). At 3 and 4 hours after irradiation, there is very strong diffuse absorption in the cytoplasm and little ultraviolet-light absorption in the nucleus; the ultraviolet-light-absorbing clumps in the nuclei have almost disappeared. Some ultraviolet-light-absorbing debris is visible (3 animals, fig. 3). At 6 hours there is more debris and less intense light absorption in the cells, both in nuclei and cytoplasm (1 animal). At 1 day after irradiation, the generative zone contains only a few large cells which absorb ultraviolet light very weakly (both nucleus and cytoplasm). The nuclei show no structure. There is much debris, and numerous leukocytes are visible (2 animals, fig. 4). At 2 and 3 days, the generative zone has almost disappeared. What cells remain contain no visible material absorbing at 263 mμ (3 animals, figs. 5 and 6).

Animals irradiated with 600 r.—At 10 and 30 minutes after irradiation, changes in 263 mμ absorption are small in nucleus and cytoplasm (4 animals). At 3 hours, the contrast between nucleus and cytoplasm is definitely lessened but the over-all level of light absorption does not seem to be markedly increased (1 animal). At 4 and 6 hours (2 animals, fig. 7), and at 1 day (3 animals, fig. 8), the changes are very similar to those observed after 2000 r. At 2 days after irradiation the gross morphological changes are much less severe than those observed after 2000 r. There are many cells in the generative zone. However, those cells do not contain much 263-mμ-absorbing material and show little contrast between nucleus and cytoplasm (2 animals, fig. 9).

At both dosage levels used, the resulting changes in 263 mμ absorption are remarkably uniform.

Discussion.—It is known that irradiation reduces both the deoxyribonucleic acid (DNA) content and the rate of DNA incorporation of the small intestine (*11–15*). By ultraviolet microscopy, one can see that changes occur in *all* generative cells; the light-absorbing clumps in the cell nuclei—presumably chromatin—disappear. This cannot be due to inhibition of DNA synthesis alone; it must be due either to degradation or leaching out of nucleic acid from the structures, or to a breaking-up and fine dispersion of the structures themselves. It is notable that the changes in 263 mμ absorption occur very uniformly in all cells of the generative zone; thus, they cannot be associated with a cell attempting to go into mitosis. Yet, there must be some physiological mechanism involved since at the doses used, only about 1 in 1,000 nucleic acid molecules is directly affected by the radiation, and even a large indirect effect mediated by the solvent could not make up the deficit.

The loss of localized 263 mμ absorption in the nucleus is accompanied

by greatly increased diffuse absorption in the cytoplasm. Mitchell, working with the squamous-cell carcinoma and adjacent epithelium, ascribes this effect to an increase in ribonucleic acid (RNA) (*16*). In intestinal epithelium, both Tillotson and Warren (*5*) and Montagna and Wilson (*17*) find that RNA is decreased. In this case, the increased cytoplasmic absorption is probably due to nonstainable components of nucleic acid, which conceivably result from the degradation of the nuclear DNA and cytoplasmic RNA. The findings of strongly absorbing debris and, somewhat later, numerous leukocytes, seem to indicate the method of disposal of degraded nucleic acid.

One day after irradiation, both ultraviolet microscopy and other cytochemical methods (*5*, *17*) indicate a considerably lowered nucleic acid content; what 263-mμ-absorbing material there is, is rather diffusely distributed and not concentrated in small clumps in the nucleus as in the normal cells. This occurs not only after irradiation with 2000 r, when it is known that cell production has been permanently blocked, but also after irradiation with 600 r, at times when cell production is about to start or has started (*4*) and the rate of DNA synthesis has returned to an approximately normal value (*11*). It is known, however, that cells produced at that time are functionally defective (*18*).

Inasmuch as successful reproduction depends on the ability of the cell to synthesize DNA, it is tempting to associate observed changes in nucleic acid metabolism with the blocking of cell production. However, our observations show no simple connection. The changes in amount and distribution of 263-mμ-absorbing material occur soon after irradiation in all generative cells simultaneously, after a time considerably shorter than the generation time, and therefore cannot be related to the mitotic cycle. At a later time, visible changes persist equally in cells in which reproduction is permanently blocked and in cells in which it has already begun.

The changes we observed are not specific for the irradiated intestine, as is demonstrated by similar changes found in 1 out of 5 nonirradiated controls. A single attempt was made to determine whether any nonspecific stress could cause a similar reaction: One mouse was forced to swim in cold water to exhaustion and was sacrificed 1 hour later; its crypts resembled those of the controls rather than the irradiated animals.

Principal Cells: Primary and Secondary Response

Photographed at 263 mμ, the principal cells of the intestinal epithelium show a nucleus containing several strongly absorbing small clumps, a moderately strong absorbing cytoplasmic structure between nucleus and surface, and a cuticular border. One hour after irradiation, the clumps in the nuclei appear less well defined, the cytoplasm absorbs light more strongly, and the contrast between nucleus and cytoplasm is lessened. This is the same type of change that has been observed in the generative cells but it is less severe; it also is less persistent, and 3 to 6 hours after irradiation the appearance is again normal. At this time, the crypts

show marked changes; accordingly, the boundary between generative and functional zone shows very clearly.

One day after irradiation, the nuclei begin to appear enlarged, ballooned, and have poorly defined internal structure. This change is intensified after 2 or 3 days, particularly with 2000 r. With the latter dose, one sees round objects in the villus cells at 3 days after irradiation, which absorb strongly at 240 mμ, indicating that they are lipide droplets. Accordingly, ether extraction was tried and found to result in disappearance of the objects. This confirms Montagna and Wilson's observation of fat droplets in the villus cells (*17*).

Discussion.—The principal cells are highly radioresistant. The changes observed 1 hour after irradiation are interpreted as primary changes; they are slight and transient. The degenerative changes which begin to appear at 1 day after irradiation are probably not primary radiation effects. At this time we are looking at cells that have arisen from irradiated crypt cells and probably have not developed normally.

Paneth Cells: Resistant Cells with a Highly Sensitive Cytoplasmic Component

The Paneth cells are a group of differentiated cells, thought to be secretory elements, located at the bottom of the crypts. They contain a cytoplasmic structure that looks like a network of channels with granules embedded in the walls; it is clearly visible with all 3 wavelengths employed (fig. 1). The Paneth cells are comparatively radioresistant; Montagna and Wilson (*17*) found them unchanged after irradiation with up to 1000 r, although Bloom (*19*) reports changes in staining properties appearing 2 hours after irradiation and lasting 9 days. The Paneth cells participate in the nuclear and cytoplasmic changes in 263 mμ absorption observed in the other crypt cells, but they do not disappear after irradiation as do the generative cells. Two days after irradiation with 2000 r, when the latter are virtually gone, the crypts are largely reduced to nests of Paneth cells (figs. 5 and 6). The Paneth cells disappear, if at all, at the same time as the last principal cells on the villi, about 3 days after irradiation with massive doses.

In spite of their over-all resistance to irradiation, it is the Paneth cells which show the earliest clear-cut changes in the ultraviolet picture. Half an hour after irradiation at either of the doses used (600 and 2000 r) the cytoplasmic network is partly transformed into isolated spherical objects with light-absorbing granules embedded in their surface. Smaller doses (200 and 300 r) and shorter times (10 minutes after irradiation) were tried; the onset of the transformation could be seen 10 minutes after treatment with 300 r (fig. 10). The disintegration of the cytoplasmic network into isolated spherical objects is fairly complete within 3 or 4 hours after irradiation; the spherical bodies are seen as debris expelled into the lumen of the crypts. Note that there are two sources of debris; the Paneth cells and, as described earlier, the generative cells.

At 1 and 2 days after irradiation, spherical bodies and debris are seen in the Paneth cell region, but there is also evidence of regeneration of a fairly normal-looking cytoplasmic network (figs. 4, 8, and 9).

Discussion.—The Paneth cells, which are highly radioresistant if survival is taken as a criterion, contain a highly sensitive cytoplasmic structure which shows radiation damage very early after administration of relatively small doses. The degeneration and regeneration of this structure is not synchronized with the response of the whole cell. This is a remarkable instance of dissociated radiosensitivities within a single cell.

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All figures are of mouse jejunum (lower two thirds of crypts), unstained sections, as photographed with wavelength 263 μ. × 450

PLATE 81

FIGURE 1.—Control. Absorbing granules in nuclei, little absorption in cytoplasm Paneth cells on bottom of crypts show cytoplasmic structures, probably secretory apparatus.

FIGURE 2.—2000 r, 1 hour after irradiation. Increased and more diffuse absorption in all cells. Changes very uniform. Cytoplasmic structure in Paneth cells largely disintegrated.

FIGURE 3.—2000 r, 4 hours after irradiation. Uniform, very intense, diffuse absorp tion; debris.

FIGURE 4.—2000 r, 1 day after irradiation. Few generative cells, little absorption; debris. Cytoplasmic structure in Paneth cells largely regenerated.

FIGURES 5 AND 6.—2000 r, 2 days after irradiation. Generative zone has virtually disappeared. Remaining cells (at bottom of crypt) are Paneth cells with fairly normal cytoplasmic structure.

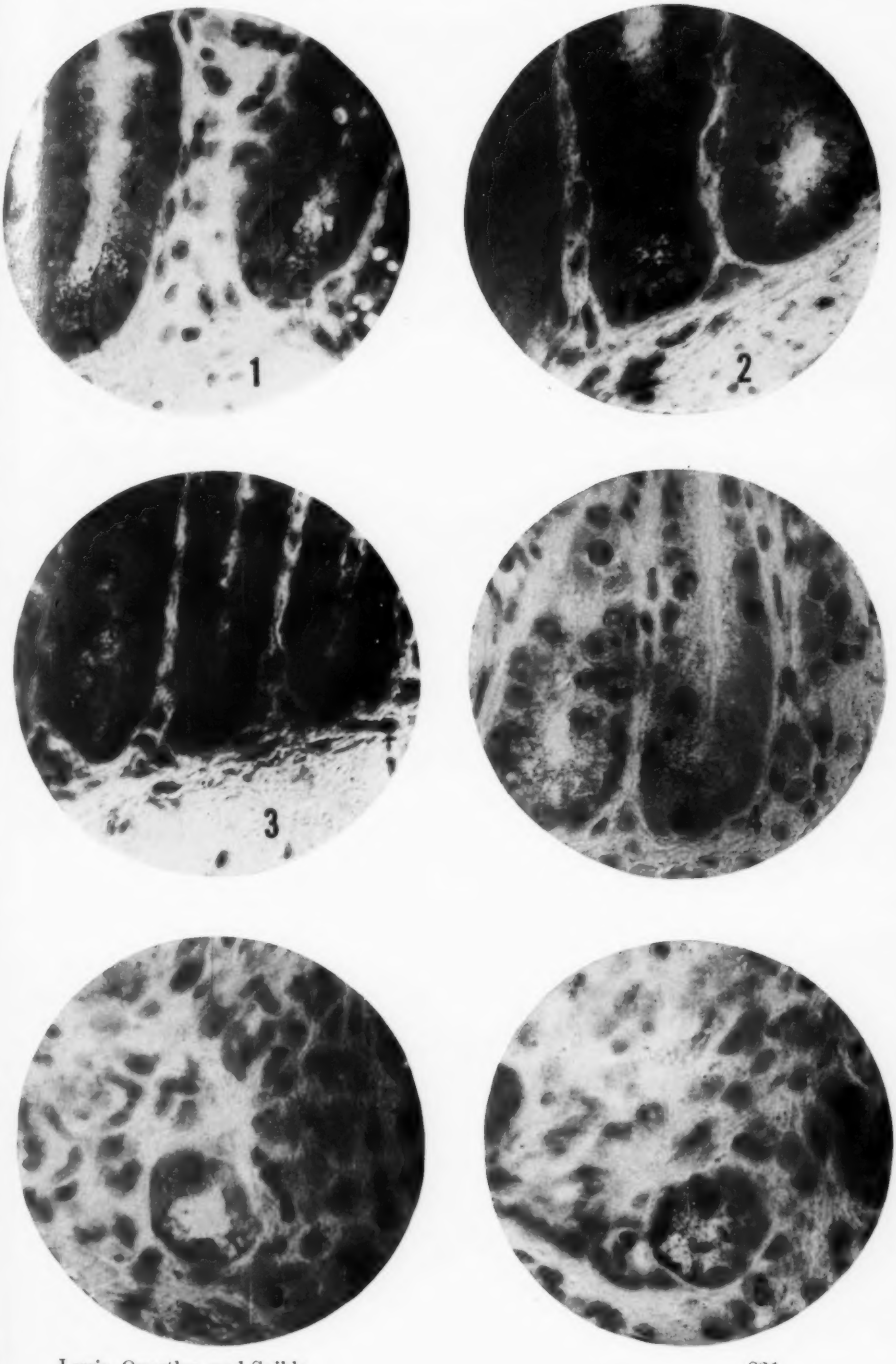

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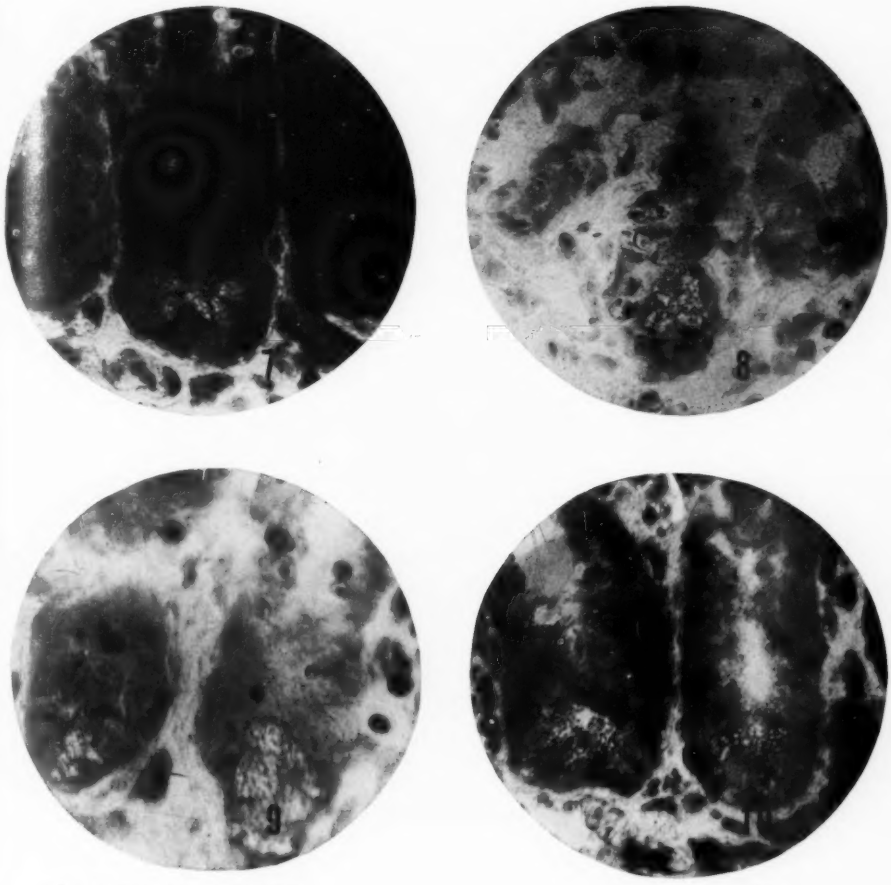
PLATE 82

FIGURE 7.—600 r, 4 hours after irradiation. Compare with figure 3.

FIGURE 8.—600 r, 1 day after irradiation. Compare with figure 4.

FIGURE 9.—600 r, 2 days after irradiation. Generative zone contains more cells than after 2000 r (figs. 5 and 6) but they show little absorption.

FIGURE 10.—300 r, 10 minutes after irradiation. Beginning disintegration of cytoplasmic structure in Paneth cells.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A Retrospective Study of Lung Cancer in Women [1,2]

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Summary

In a controlled, retrospective investigation of 158 women with pulmonary carcinomas, the largest and the only statistically significant effects were associated with smoking history. The scale of relative risks by intensity of cigarette use was greater for epidermoid and undifferentiated carcinomas than for adenocarcinomas. For epidermoid and undifferentiated carcinomas all the relative risks, with respect to smoking history and rate of cigarette use, differed significantly from unity at the 0.1 percent level. The findings agree substantially with those from three other studies of lung cancer in women. The combined results of several investigations suggest that the characteristic excess lung-cancer mortality among males almost disappears when nonsmokers are studied, since male nonsmokers have only slightly higher rates than female nonsmokers.—**J. Nat. Cancer Inst. 21: 825–842, 1958.**

One of the striking characteristics of pulmonary carcinoma is its pronounced excess risk among males when compared with the low incidence among females. This sex disparity has led to postulations regarding the role of hormones and other sex-linked differences in the etiology of lung cancer and has been referred to as one of the inconsistencies in the strong relationship between the smoking of cigarettes and lung cancer, which has been demonstrated in a series of investigations on males (*1–8*). Specifically the suggestion has been made that sex differences in duration and rate of cigarette use have not been large enough to account for either the sex differences in lung-cancer mortality trends or in the current death rates (*9, 10*).

These speculations have not been documented as yet by a detailed examination of the available evidence. Data suggesting that sex differences in lung-cancer risk might be reconciled if smoking history were taken into account have been reviewed (*11*). In an earlier communication

[1] Received for publication May 16, 1958.

[2] Part of this paper was presented the week of July 6, 1958, at the Seventh International Cancer Congress in London.

[3] National Institutes of Health, Public Health Service, U. S. Department of Health, Education, and Welfare.

(*12*) we applied a schedule of relative lung-cancer risks for smokers and nonsmokers, established by forward and retrospective studies among men, to population survey data on smoking habits. The results suggested that lung-cancer mortality among women is not inconsistent with the findings established by observation of men, and that adjustment for cigarette-smoking history brings the sex ratio for lung-cancer mortality into close conformity with that for other causes. Apart from the proper interpretation of the facts, the results suggest the important part, entirely disproportionate to the numbers involved, that studies among women may have in working out the epidemiology of lung cancer. Only three papers reporting on sizable numbers of female patients have been published (*2*, *13*, *14*). Further studies are needed to establish more precisely, by direct observation among women, the variation in lung-cancer risk by smoking history and other characteristics. The controlled series, presented here, of 158 female patients with microscopically confirmed primary pulmonary carcinoma is substantially larger than any hitherto assembled.

Materials and Methods

To assemble a sufficient number of case histories within a reasonable period, arrangements were made for collection of data from several cooperating institutions. Returns have been secured from 29 such sources; the largest number of case histories supplied by a single source was 40. Only patients available for interview were eligible for inclusion in this series. Most of the histories were obtained by a resident in the hospital to whom a small honorarium was paid (in some hospitals interviews were conducted by a medical social worker or tumor-clinic secretary). Since the interviewers were part of the hospital staff, it was not usually feasible to conduct "blind" interviews without knowledge of the identity of the patient and controls. The completed histories and supporting diagnostic records were sent to the Biometry Branch, National Cancer Institute where they were reviewed for completeness and consistency. We wish to emphasize that the analyses and the conclusions reached are the responsibility of the authors and do not necessarily represent the opinions of the clinicians or institutions contributing individual case histories.

The decentralized approach to data collection required that the questionnaire and procedures for selecting controls be kept as simple as possible. The questionnaire covered place and date of birth, present address, race, usual occupation, marital and childbearing history, smoking habits, and, as an attempted control question, coffee use. The two controls for each patient with a diagnosis of cancer were women in the same hospital and service at the time of the interview; one being the next older woman and the other the next younger. The rule was modified for some hospitals with a separate chest-surgery service where controls were drawn from general surgery or medical services to reduce the representation of tuberculosis diagnoses. Age was the only variable on which matching of cancer patients and controls was attempted.

Collection of data began in March, 1955, and this report covers lung-cancer patients interviewed during the subsequent 2-year period. The instructions stressed that interviews should not await final confirmation of the diagnosis. Of the first 194 records submitted, 12 were eliminated because a diagnosis other than lung cancer was subsequently reached, and 16 because no microscopic confirmation was available. Six patients with alveolar cell carcinoma and 2 with bronchial adenoma also were excluded. Table 1 distributes the remaining 158 patients by the final determination of histologic type (adenocarcinoma, epidermoid and undifferentiated carcinoma) and nature of the diagnostic evidence.

TABLE 1.—*Histologic type and basis for diagnosis of female lung-cancer patients*

Basis for diagnosis	Total	Adeno-carcinoma*	Epider-moid carcinoma	Undifferen-tiated carcinoma†
Total	158*	56	42	60
Autopsy	26	7	7	12
Tissue from primary site	108	38	32	38
Smear from primary site	6	2	1	3
Tissue from metastatic site	12	4	1	7
Tissue from metastatic site (lung not established unequivocally as primary site)	6	5	1	—

* Excludes 6 cases of alveolar cell carcinoma and 2 of bronchial adenoma.
† Includes "anaplastic," "oat cell," and "small cell."

Some analyses were carried out for the 134 patients on whom autopsies or microscopic examinations of tissue from the primary site were performed. The latter results agreed substantially with those for the more inclusive group of 158 patients, indicating that none of the effects reported has been produced by the inclusion of the few patients whose diagnostic evidence was less positive.

Differences of opinion exist among pathologists on the histologic classification of pulmonary carcinomas, particularly in relation to epidermoid and undifferentiated carcinomas. This disagreement was quite evident in returns from individual hospitals, among which the ratio ranged from such extremes as 9 epidermoid and 3 undifferentiated to 1 epidermoid and 13 undifferentiated. On the other hand, the proportion of adenocarcinomas was stable; the results for individual hospitals did not differ significantly from the pooled experience of all institutions. Because of the fluid opinion on classification of epidermoid and undifferentiated carcinomas, it was felt that arrangements for further pathologic review would not be worth while. It may be noted here that the major finding of an association with smoking history holds for the combined group of women with pulmonary carcinomas, and that detection of this gross effect does not depend on a subclassification by histologic type.

The women with epidermoid and undifferentiated carcinomas resembled each other in most characteristics for which data were collected. The age distributions for these women (epidermoid and undifferentiated cases)

were similar (table 2) and their combined proportion under 55 years was 45 percent, which was somewhat larger than the 34 percent figure for adenocarcinomas. In this analysis the epidermoid and undifferentiated types have been pooled. This grouping resembles Kreyberg's Group I lung tumor classification, which includes squamous-cell, large cell, and small cell carcinomas (*15*). (Kreyberg's Group II includes adenocarcinomas, bronchiolar carcinomas, adenomas, and salivary-gland tumors.) If there are differential effects associated with epidermoid and undifferentiated types, grouping would tend to dilute them so they might escape detection. Some analyses were carried out separately for the epidermoid and undifferentiated groups, but these revealed no consistent, systematic differences and have not been presented here.

TABLE 2.—*Age distribution of patients (diagnosed cases) and controls*

Age	Adenocarcinoma		Epidermoid carcinoma		Undifferentiated carcinoma		Controls	
	Number	Cumulative percent	Number	Cumulative percent	Number	Cumulative percent	Number	Cumulative percent
Total	56	—	42	—	60	—	339	—
Under 45	8	14.3	6	14.3	8	13.3	51	15.0
45–54	11	33.9	13	45.2	19	45.0	94	42.8
55–64	27	82.1	10	69.0	21	80.0	117	77.3
65 and over	10	100.0	13	100.0	12	100.0	77	100.0

If the determination of histologic type is influenced in part by the nature of the specimen examined, the interpretation of the relative risks for histologic-type groups could be complicated by indirect association of some characteristics with methods of diagnosis such as the use of bronchoscopy. Gilliam has deduced from indirect evidence that resections yield a lower proportion of epidermoid and undifferentiated carcinomas than do biopsy specimens (*16*). The present data support that deduction. Fifty-three percent of the resected patients (without autopsy) were reported in the epidermoid or undifferentiated class, while the figure for patients with biopsy, alone, was 77 percent. Among those autopsied, the proportion was 73 percent. When tumors are of a mixed type there is more opportunity for larger resection specimens to show evidence of adenocarcinoma. However, the correspondence between the autopsy and biopsy results suggests the possibility of some selection among resected patients, those with biopsies indicating epidermoid or undifferentiated types being less likely candidates for this procedure. Selection might be anticipated, since some chest surgeons subscribe to the statement that ". . . a biopsy that demonstrates highly undifferentiated or oat cell cancer is presumptive evidence that complete surgical extirpation is a remote possibility . . ." (*17*), although this view on the prognostic value of histologic type is not supported by Overholt's data (*18*). The association

observed between histologic type and nature of specimen would not appear to be a serious classification artifact and, while the occurrence of some errors in histologic classification must be admitted, we do not feel they would vitiate the results presented in table 5.

Controls.—Since only one woman with lung cancer was under 35, controls under 35 years were discarded in the analysis, leaving a total of 339 controls. The diagnoses for the controls are presented in table 3. Roughly, three tenths had cancer of sites other than the respiratory system. A variety of diseases reflecting the experience encountered in surgical and medical services accounted for the remainder. The simplicity of the rule for identifying and selecting inpatients for interview made this approach preferable to sampling admitted patients with no guarantee that persons selected would be available for interview in the hospital. There is a possibility that controls drawn from a patient population overrepresent diagnoses of patients requiring longer hospital stays and may not reflect accurately the distribution of admission diagnoses (*19*). However, this possible bias could not have been an important factor since trial tabulations indicated that exclusion of selected diagnostic categories from the controls did not materially change the results.

TABLE 3.—*Diagnoses for female controls*

Total	339
Cancer of sites other than respiratory system (140–159, 170–205)*	96
Breast (170)	23
Genital organs (171–176)	20
Lymphosarcoma, etc. (200–203, 205)	12
Leukemia (204)	5
Other sites	16
Cardiovascular-renal diseases (330–334, 400–468, 592–594)	50
Diseases of the respiratory system (470–527)	40
Diseases of the digestive system (530–553)	31
Benign neoplasms (210–229)	21
Allergic, endocrine system, metabolic, and nutritional diseases (240–289)	19
Accidents (800–999)	11
Tuberculosis (1–19)	10
Other diagnoses	31
Symptoms, ill-defined conditions, diagnosis not established at time of interview	30

*International List Numbers, sixth revision, shown in parentheses.

The representative nature of the hospital controls may be assessed in part by comparing some of their characteristics with data available for United States women. After appropriate adjustments for age, the hospital controls had distributions similar to those for the general population with respect to marital status, childbearing history, and proportion currently residing in State of birth. Allowing for underrepresentation of study material from the Northeast and North Central States, we also found close to the expected number of nonwhites and foreign born. These checks do not suggest unusual selection among the hospital controls.

Table 4 compares tobacco use among the hospital controls with that reported for women in the general population. The proportion who "never smoked" was definitely lower among the hospital controls, particularly for women under 55. Compensatory differences appeared among "current regular cigarette smokers." Our control result for "nonsmokers" agreed well with the hospital female control experience reported by Wynder (*14*) and by Watson and Conte (*13*). The same type of relationship between hospital and general population controls has appeared in retrospective studies of males.

TABLE 4.—*Tobacco use among hospital controls compared with that reported for a sample of U. S. women*

Age	Percent "never smoked"		Percent "current, regular cigarette smokers"	
	Hospital controls	U. S. women, 1955 (*20*)	Hospital controls	U. S. women, 1955 (*20*)
35–44	45	58	45	32
45–54	57	69	35	23
55–64	80	83	16	11
65 and over	88	93	5	4

Other investigators have reported an association between smoking history and coronary artery disease and diseases of the respiratory system other than lung cancer (*21–24*). The inclusion of these diagnoses, which are not a numerically important component of our control series, was not responsible for the lower proportion of nonsmokers among the hospital controls as compared to the general population controls. Exclusion of these diagnoses from the hospital control series left the over-all proportion "never smoked" unchanged at 70 percent. In this paper the conservative course of using only hospital controls for the estimation of relative risks has been followed. The use of general population controls would yield higher estimates of lung-cancer risk among women smokers.

Results

The results appear in table 5 as relative risks, *i.e.*, estimates of the probability of lung cancer developing in persons with a specified characteristic in terms of a unit risk for persons without that characteristic. A presentation oriented to estimates of magnitude of effects as well as to tests of significance was desired, since decisions on the meaning to be attached to any effect usually depend on the collation of evidence from several sources rather than on a result from a single study. Some of the larger risks for certain characteristics, even though not statistically significant here, may be suggestive when examined in conjunction with data from other studies.

Relative risk estimates have been adjusted statistically to control for differences between cancer patients and controls with respect to other

TABLE 5.—*Estimated relative risk* of lung cancer by histologic type for several population characteristics*

Contrast†		Adjusted for	Relative risk*	
			Epidermoid and undifferentiated carcinomas	Adenocarcinoma
Smoking History				
History of any tobacco use (58,18,103)	*vs.* no tobacco use (44,37,236)	Age	3.0‡	1.21
		Age, occupation	3.0‡	1.19
		Age, amount of coffee	2.6‡	1.14
Current Cigarette Use				
21+ cigarettes daily (18,5,13)	*vs.* never used cigarettes (46,37,236)	Age	7.1	2.8
20 cigarettes or less daily (30,10,66)			2.3‡	1.07
Occasional or discontinued smoker (8,3,24)			1.69	0.84
		Age, occupation	6.3	3.5
			2.3‡	1.04
			1.70	0.75
		Age, amount of coffee	5.6	2.4
			2.1‡	1.04
			1.61	0.76
Maximum Cigarette Use				
Maximum rate 21+ cigarettes daily (19,5,17)	*vs.* occasional or nonsmoker (51,38,251)	Age	5.5‡	2.2
Maximum rate 20 or less cigarettes daily (32,12,71)			2.2	1.25
		Age, occupation	4.8‡	2.8
			2.2	1.17
Maximum rate 21+ cigarettes daily (19,5,17)	*vs.* maximum rate 20 or less cigarettes daily (32,12,71)	Age, years at maximum rate	2.2	1.65
		Age, age started smoking	2.2	2.3

See footnotes at end of table.

TABLE 5.—*Estimated relative risk* of lung cancer by histologic type for several population characteristics*—Continued

Contrast†		Adjusted for	Relative risk*	
			Epidermoid and undifferentiated carcinomas	Adenocarcinoma
Age Started Cigarette Smoking				
Started cigarette smoking under age 25 (24,6,37)	*vs.* never smoked (46,37,236)	Age	3.4	1.12
Started cigarette smoking over age 25 (32,12,66)	*vs.* never smoked (46,37,236)	Age	2.5‡	1.27
		Age, occupation	3.3	1.10
			2.4‡	1.25
Started cigarette smoking over age 25 (32,12,66)	*vs.* started cigarette smoking under age 25 (24,6,37)	Age, current cigarette use	0.92	0.95
Duration of Cigarette Use				
Smoked cigarettes 15+ years (42,16,77)	*vs.* never smoked (46,37,236)	Age	2.8	1.45
Smoked cigarettes <15 years (14,2,26)	*vs.* never smoked (46,37,236)	Age	2.8‡	0.54
Smoked cigarettes 15+ years (42,16,77)	*vs.* smoked cigarettes <15 years (14,2,26)	Age, current cigarette use	0.81	1.95
Duration of Maximum Cigarette Use				
Smoked at maximum cigarette rate for 15+ years (39,13,66)	*vs.* never smoked (46,37,236)	Age	3.0	1.38
Smoked at maximum cigarette rate for <15 years (17,5,37)	*vs.* never smoked (46,37,236)	Age	2.4‡	0.94
Smoked at maximum cigarette rate for 15+ years (39,13,66)	*vs.* smoked at maximum cigarette rate for <15 years (17,5,37)	Age, maximum cigarette use	1.19	1.42
Coffee Drinking				
4+ cups daily (28,10,63)	*vs.* no coffee (12,6,52)	Age	1.93	1.32
1–3 cups daily (62,39,224)	*vs.* no coffee (12,6,52)	Age	1.21	1.40
		Age, tobacco use	1.38	1.13
			1.20	1.32
		Age, current cigarette use	1.13	0.89
			1.05	1.08

Black coffee (34,19,93)	*vs.* coffee with cream (56,30,194)	Age, amount of coffee	1. 23	1. 27
		Age, amount of coffee, tobacco use	1. 02	1. 23
		Age, amount of coffee, current cigarette use	1. 02	0. 93
Occupation				
White collar workers (38,13,88)	*vs.* never worked (40,26,153)	Age	1. 65	0. 89
Nonwhite collar workers (24,16,98)			0. 94	1. 02
		Age, history of tobacco use	1. 42	0. 83
			0. 79	0. 92
		Age, current cigarette use	1. 27	0. 83
			0. 80	0. 94
Worked 10+ years (45,19,129)	*vs.* never worked (40,26,154)	Age	1. 33	0. 92
Worked <10 years (17,10,57)			1. 19	1. 08
Worked 10+ years (45,19,129)	*vs.* worked <10 years (17,10,57)	Age, occupation	1. 19	0. 81
Migration				
Native-born migrants (13,10,46)	*vs.* native-born nonmigrants§ (82,38,265)	Age	0. 89	1. 72
Foreign-born (7,7,28)			0. 80	1. 66
		Age, current cigarette use	0. 77	1. 47
			1. 01	1. 76
Marital Status				
Married once (77,43,250)	*vs.* never married (11,5,31)	Age	0. 89	1. 07
Married more than once (14,7,58)			0. 69	0. 77
Pregnancy History				
Married nulliparae (17,6,42)	*vs.* single nulliparae (11,4,30)	Age	1. 14	1. 02
1–4 pregnancies (49,32,198)			0. 69	1. 23
5+ pregnancies (25,13,69)			1. 01	1. 38
1+ stillbirths (32,12,107)	*vs.* no stillbirths (34,27,141)	Age, number of pregnancies	1. 12	0. 63
Twin pregnancy (6,3,11)	*vs.* no twin pregnancy (68,42,256)	Age, number of pregnancies	1. 65	1. 39
Race				
Nonwhites (15,7,40)	vs. whites (87,48,299)	Age	1. 28	1. 03

*In terms of unit risk for last-named group in each contrast.

†Respective numbers of epidermoid and undifferentiated carcinomas, adenocarcinomas, and control observations in each contrast group are indicated in parentheses.

‡Double dagger indicates relative risks significantly different from unity at the 0.1 percent level. None of the remaining relative risks was significantly different from unity at the 5 percent level.

§Still living in state or region of birth.

factors specified. All comparisons that held race constant and several that controlled occupation and duration of tobacco use have been omitted from table 5 since the effects of these adjustments were negligible. The computations involved subclassification of the data and the summation of observed and expected values over all subclasses. For a set of fourfold tables, in which the individual cell entries are represented by $\begin{array}{|c|c|}\hline a & b \\ \hline c & d \\ \hline \end{array}$, the formula used can be written as

$$\text{Relative risk} = \frac{\Sigma a \Sigma d}{\Sigma b \Sigma c} \div \frac{\Sigma E(a) \Sigma E(d)}{\Sigma E(b) \Sigma E(c)}.$$

The rationale underlying this formula will be discussed in another paper (*25*). In the simple situation of homogeneous groups of cancer patients and controls, represented by a single fourfold table, the formula reduces to the familiar ratio, ad/bc.

None of the other items considered—occupation, coffee drinking, migration, marital status, pregnancy history—had relative risks approaching in magnitude those associated with smoking history. Smoking history (current cigarette use, maximum cigarette use) was the only item for which there were statistically significant associations with all pulmonary carcinomas combined. Inspection of table 5 reveals that for epidermoid and undifferentiated carcinomas all the relative risks with respect to smoking history and rate of cigarette use differed significantly from unity at the 0.1 percent level, while none of the risks for other characteristics was significant at the 5 percent level. None of the relative risks shown for adenocarcinomas differed significantly from unity at the 5 percent level.

Cigarettes were the only important form of tobacco use among these subjects. The risk of epidermoid and undifferentiated carcinoma among tobacco users sharpened when the contrast was based on current rate of use. The highest risk of epidermoid and undifferentiated types (7.1:1) appeared among women using more than 1 pack daily when compared with women who never used cigarettes. The risk for occasional and discontinued smokers was higher than for nonusers. Classification by maximum rate yielded essentially the same findings as rate of current use. The lower risks for "maximum rate" contrasts arose from shifting the unit risk base to "occasional or nonsmokers." It may well be that there is no association between adenocarcinoma of the lung and smoking and that the present scale of risks really reflects the influence of inclusion of some undifferentiated carcinomas under the category of adenocarcinomas.

Both occupation and coffee drinking showed positive association with smoking history. Thus control of these items in the computation of relative risks by degree of tobacco use would generally reduce the magnitude of the risks, and this downward displacement appeared in the adjusted risks for epidermoid and undifferentiated carcinoma by smoking history and current and maximum cigarette use. The adjusted risks, however, remained large and significantly different from unit risk.

Other aspects of smoking history examined included age at which cigarette smoking was begun, duration of use, and duration of maximum rate of use. Women who started smoking at ages under 25 had a higher risk of epidermoid and undifferentiated carcinoma than those who began later in life; this effect disappeared when current rate of use was controlled. Control of rate of use also eliminated the higher epidermoid and undifferentiated risk among women using cigarettes for 15 years or more. Wynder's study also failed to uncover an independent effect associated with duration of cigarette use (*14*). However, among women, the association of starting age and duration of tobacco use with current rate is so strong that it may be unrealistic to expect to find a separate duration effect in retrospective studies of limited size.

Coffee drinking was included as a control item. In view of an anticipated association between smoking and coffee use, we were interested in examining the behavior of relative risk estimates when smoking history was controlled in coffee-drinking contrasts, and *vice versa*. A large but not statistically significant risk (more pronounced for epidermoid and undifferentiated carcinoma) appeared for persons consuming 4+ cups of coffee daily. The effects were diminished and virtually disappeared when history of tobacco use and current cigarette use were successively controlled in the amount of coffee-drinking contrasts. The pattern was repeated in contrasts of black-coffee drinkers *versus* those using cream. This behavior differs sharply from the control item of coffee drinking in tobacco-use contrasts in which smokers remained at high risk after adjustment for coffee drinking. Evidently the effects attached to smoking history are more potent than those attached to coffee use, and the latter factor can be ruled out as a possible explanation for major effects associated with smokers.

There were no significant results for occupation, and the relative risk of epidermoid and undifferentiated carcinoma among white-collar workers was reduced to unimportant dimensions when current rate of cigarette use was controlled. This agrees with Wynder's finding of no occupational effect in his series of studies (*14*).

Examination of migration history was suggested by Eastcott's demonstration of excess lung-cancer mortality in New Zealand among immigrants from Great Britain (*26*). Since within the United States the major stream of migration for the past half century has been from rural areas to metropolitan centers, where lung-cancer rates are higher, some additional effects might have been anticipated from the migration contrasts. While no significant finding emerged from the present data, the results were unusual in suggesting an effect for adenocarcinoma. Nothing has been reported that would lead one to suspect a higher adenocarcinoma risk among migrant women. Whether an exception to the general observation of greater variation in rates for epidermoid and undifferentiated pulmonary carcinomas, rather than for adenocarcinomas, might be uncovered by further pursuit of this line of investigation is debatable.

No striking effects were found for marital status or pregnancy history,

which is a result not inconsistent with Lilienfeld's data on marital status and pregnancy history for a limited series of female lung-cancer patients (*27*), and it seems doubtful whether female lung cancer resembles breast and uterine cancer in this respect. If there are strong hormonal or other sex-linked effects for female lung cancer, they do not carry over into these particular characteristics in the present data.

The absence of an effect associated with race was not unexpected. While the 10-city morbidity survey reported incidence to be 12 percent higher among white women (*28*), a retrospective study on the present scale could not be expected to detect so small a difference.

Discussion

Our findings agree with 3 other investigations of lung cancer in women in pinpointing a major effect associated with a history of smoking cigarettes, particularly for neoplasms of the epidermoid and undifferentiated histologic types. The 4 studies among women display conformity in several respects despite their limited scope and differences in type of populations covered. Table 6 lists some of the comparable findings. The frequency of adenocarcinomas in our series checks with Wynder's results. The 3 American studies give consistent answers on the proportion of nonsmokers among those with diagnosed cases and among controls. All indicate a lower proportion of nonsmokers among patients with epidermoid and undifferentiated carcinomas in the presence of close correspondence between those with adenocarcinomas and the controls. The fewer adenocarcinoma cases and the more smokers in the Doll-Hill study may reflect real differences in English and American experience.

The consistency of the pattern emboldens us to go beyond computations of relative risk and to attempt estimates of absolute lung-cancer mortality among female and male *nonsmokers* similar to those previously essayed by Doll from more limited data (*29*). This approach offers a most direct

TABLE 6.—*Comparison of data on histologic type and smoking history reported in 4 controlled retrospective studies of lung cancer among women*

	Microscopically confirmed cases		Percent of nonsmokers among			
	Number	Percent classified as adenocarcinoma	All cases	Adenocarcinomas	Not adenocarcinoma*	Controls
United States:						
Present study	158	35	51(59)†	66(71)	43(53)	69(77)
Wynder *et al.* (*14*)	105	39	56	71	38	74
Watson and Conte (*13*)	36	—‡	58	—‡	—‡	82
England and Wales:						
Doll and Hill (*2*)	79	13	37	50	35	55

*Includes "epidermoid," "undifferentiated," and "anaplastic," but excludes carcinomas of unclassified type.

†Two results on the proportion of nonsmokers are shown for the present study—one based on lifetime history and the other, in parentheses, reflecting status before onset of illness. The data of Wynder *et al.* and Doll and Hill refer to lifetime history. The Watson and Conte determination of smoking status was not stated.

‡Data not reported by histologic type.

sex comparison, which is free of such possible complications as differences in age when smoking was started, duration and intensity of exposure, and biologic response to tobacco smoke. The top half of table 7 summarizes the computations for women. The estimates projected from the 3 American studies range from 3.3 to 3.9 deaths, annually, per 100,000 female nonsmokers. All the rates for males and females in table 7 may be slight overestimates (the computations include a minimum estimate for the proportion of nonsmokers among controls provided by hospital rather than general population data) but this would not affect the general comparability of the results. In the absence of published data by histologic type, the rates refer to total lung-cancer mortality.

TABLE 7.—*Summary of results of retrospective studies on estimates of lung-cancer risk among nonsmokers*

Studies	Time period covered	Nonsmokers (*percent*)		Estimated risk among nonsmokers in terms of unit risk for total population (p_1/p_2)	Estimated mortality rate per 100,000 nonsmokers*
		Cases (p_1)	Controls (p_2)		
Females					
Present study	1955–57	59. 5	76. 7	. 78	3.9 (U. S., 1955)
Wynder *et al.* (*14*)	1953–55	56. 2	74. 4	. 76	3.8 (U. S., 1955)
Watson and Conte (*13*)	1950–52	58. 0	82. 0	. 71	3.3 (U. S., 1950)
Doll and Hill (*2*)	1948–51	37. 0	54. 6	. 68	4.6 (England & Wales 1951)
Males					
Sadowsky *et al.* (*5*)	1938–43	3. 8	13. 2	. 29	3.0 (U. S., 1940)
Mills and Porter (*4*)	1940–46 for cases, 1947 for controls	7. 0	31. 0	. 23	3.3 (U. S., 1945)
Levin (*30*)	1938–52	8. 1	27. 1	. 30	4.3 (U. S., 1945)
Wynder and Graham (*7*).	not stated, published in 1950	1. 3	14. 6	. 09	2.1 (U. S., 1950)
Breslow *et al.* (*1*)	1949–52	3. 7	10. 8	. 34	7.9 (U. S., 1950)
Watson and Conte (*6*)	1950–52	1. 9	9. 7	. 20	4.6 (U. S., 1950)
Doll and Hill (*2*)	1948–51	0. 5	4. 5	. 11	5.1 (England & Wales, 1951)

*The male and female lung-cancer mortality experience (age adjusted to U. S. census population of 1950) used in deriving the estimate for nonsmokers is shown in parentheses.

Additional estimates were projected from the larger retrospective studies among males, which appear in the latter half of table 7. The male estimates range from 2.1 to 7.9 per 100,000 nonsmokers, with an average value of 4.2. The smaller number of nonsmokers among men subjects the estimated rates for that sex to greater variability. The studies among men span a longer interval and exhibit a downward trend in the proportion

of nonsmokers among cancer patients and controls in keeping with other information on increased tobacco use (*20*), and it seemed desirable to relate the observations to the mortality prevailing during the study period. It would be unwise to attempt inferences from these data on lung-cancer mortality trends among male nonsmokers.

Using the data reported from the forward study of white males by the American Cancer Society, Inc. (*24*), we derived an estimated lung-cancer mortality rate of 5.7 per 100,000 nonsmokers. The men studied were presumably well, at initial interview, so that the mortality observed would be lower than that prevailing in the corresponding general population. Our estimate was obtained by noting that the lung-cancer rate for nonsmokers was 20.9 percent of that for the total study population and by applying this factor to the 1955 age-adjusted rate for United States males.

Considering the biases to which individual studies may be subject, the concordance of the estimated mortality rates seems surprisingly good. The evidence suggests a modest excess risk for male nonsmokers, no greater than, and possibly less than, the 40 percent excess male mortality encountered at ages over 35, in the United States, for all causes, and for such groups of causes as pneumonia, influenza, and cancer of all sites other than the respiratory system and the reproductive organs. *No sex-linked factor peculiar to lung cancer need be postulated to account for the results on nonsmokers.*

The findings appear consistent with a recent study in the North Wales and the Liverpool region, by Stocks, who concluded that "Amongst nonsmokers living continually in rural surroundings the male and female (lung cancer) death rates appear to be equal." (*31*).

The nature of the mortality relationship between male and female nonsmokers facilitates an interpretation of the relative risk results by smoking class. For example, the same relative risk schedules, by sex, for moderate and heavy smokers (based on unit risk for nonsmokers) would imply the presence of a "normal" male excess in risk over the entire spectrum of cigarette use. Table 8 summarizes some relative risk estimates for men and women, which take account of rate, but not duration, of tobacco use. Our results are supplemented by computations based on data published by Wynder *et al.* (*14*). Since adenocarcinoma is not strongly associated with smoking history, relative risks for lung cancer, excluding adenocarcinoma, are also given. The findings from forward and retrospective studies among men appear generally consistent.

The estimated risks for women are subject to large sampling errors and only the most tentative inferences can be drawn. The observations for women using over 1 pack daily are extremely limited, and neither the difference between the two female results nor their divergence from the corresponding male relative risks are statistically significant. The data indicate that, among persons smoking 1 pack of cigarettes or less daily, women have much the lower relative risks, and that the sex disparity in this regard seems more marked in the "1-pack-and-under" than in the "over-1-pack" category. This disparity is also suggested in some British

TABLE 8.—*Comparison of estimates of relative risk of lung cancer by sex and smoking class*

Smoking class	Females				Males		
	Present study		Wynder *et al.* (*14*)		Retrospective studies summarized by Cutler and Loveland (*32*)	American Cancer Society, Inc., prospective study (*24*)	
	All types	Excluding adenocarcinoma	All types	Excluding adenocarcinoma	All types	All types	Excluding adenocarcinoma
Nonsmoker	1	1	1	1	1	1	1
One-half pack or less daily	1.8	2.3	1.6	1.8	4	7	9
One-half to 1 pack daily					8	7	10
More than 1 pack daily	5	7	11	25	13	16	28

results published by Doll and Hill (*2*). Two interpretations might be advanced to account for these results. One is that sex differences in intensity and duration of use within the same smoking class are greater for light and moderate smokers as opposed to heavy smokers. (A survey of tobacco use in the United States has indicated that the smallest sex differences in age, when smoking was begun, occur among smokers attaining a maximum rate of more than 1 pack daily, *20*.) The second possibility is that occupational exposures, to which males would be more subject, might exert a more prominent effect among males exposed to light or moderate concentrations of cigarette smoke. There is certainly some evidence for the presence of occupational effects for lung cancer when smoking habits are controlled in the analysis (*1*). The data available for women seem consistent with the hypothesis of an etiologic relationship between cigarette smoking and the occurrence of epidermoid and undifferentiated carcinoma of the lung, but further studies of sex differences, in risk by histologic type, smoking class, occupation, and other factors, should be pursued to elaborate additional facets of lung-cancer epidemiology.

The disparity between epidermoid and undifferentiated carcinoma and adenocarcinoma in degree of association with smoking history reveals the fallacy of using published lung-cancer mortality data (which do not distinguish histologic types) to demonstrate inconsistencies in the cigarette-smoking, lung-cancer hypothesis by noting incommensurate sex differences, in rate of increase of total lung-cancer mortality and in rate of adoption of the smoking habit (*9*). The proportion of adenocarcinomas is substantially higher among women (*2*, *28*, *33*), and the presence of a relatively stable adenocarcinoma rate could dilute, almost beyond recognition, any increase in the incidence of epidermoid and undifferentiated types. Norwegian autopsy data assembled by Kreyberg (*15*), and presented in the form of a relative frequency of Group I to Group II tumors, suggest that differences between sexes and over a time are smaller for his

Group II tumors (which include adenocarcinomas) than for Group I tumors. Autopsy series available elsewhere and covering a period of years might be re-examined to determine the nature of any change, over a time, in the ratio of adenocarcinoma to other histologic types, by sex, to see if Kreyberg's results can be reproduced and extended.

The evidence on trends in vital statistics might be supplemented by drawing samples of lung-cancer deaths and querying the nature of the supporting diagnostic evidence and, when available, histologic type. In collaboration with the National Office of Vital Statistics we are doing this for a 10 percent sample of lung-cancer deaths in the United States for 1958. Repetition at intervals could then delineate subsequent trends by histologic types. Some of the United States comparisons over a time might be anticipated by drawing comparisons with other countries. For example, the United States rates for both men and women lag more than 10 years behind the English experience, and it would not be unreasonable to assume that current English lung-cancer mortality is a forecast of what the United States rate will be several years hence. If a parallel program of querying lung-cancer deaths for histologic type could be undertaken in England, this would permit an assessment of intercountry variation by histologic type and would provide some basis for predicting the future course of the adenocarcinoma and non-adenocarcinoma rates in the United States. To assure comparability of histologic classification, such a program would require provisions for an interchange of a sample of sections between pathologists in the two countries.

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Histocompatibility Genes of the Mouse. II. Production and Analysis of Isogenic Resistant Lines [1,2]

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Summary

The production and *H*-gene typing of isogenic resistant (IR) lines in the mouse is described. Thirty-eight lines reached a stage of development where significant information as to their genotype could be obtained; 26 of these were studied in some detail. Of the 38 original IR lines, 30 were shown to be distinguished from their inbred or "coisogenic" mate by a difference at the *H-2* locus. One coisogenic pair, C3H and C3H.K, differs at *H-1*, another, C57BL/10 and B10.LP, at *H-3*. Of the 6 remaining lines, 1 probably had an *H-1* difference, 3 were uncertain, and 2, still under test, may serve to define new loci. Two lines served to identify new *H-2* alleles: D1.C is $H\text{-}2^c$, AKR.M is $H\text{-}2^m$. The component test showed D1.C to have the D component, and AKR.M the K component. Linkage tests showed 7.1 ± 4.5 percent crossing over between *H-1* and *c*, and 9.8 ± 6.4 percent crossing over between *H-3* and *A*, both cases in heterozygous females. These values may be high. Evidence is presented that F_1 hybrids, with the tumor donor as one parent, are less compatible with tumor grafts than the native strain. With one exception, the resistance shown by the hybrid was slight. It was not increased by immunization. The extent to which the synthesized IR lines and their inbred mates approach the true coisogenic state is discussed. Table 15 presents a summary of the current state of our information in regard to the *H*-2 locus.—**J. Nat. Cancer Inst. 21: 843–877, 1958.**

In an earlier paper (*1*) we discussed the various methods available for the analysis of histocompatibility genes in the mouse. The conclusion was reached that *isogenic resistant* or IR lines would provide the best means for the identification of individual histocompatibility loci. Subsequently we borrowed from *Drosophila* workers (*2*) the term *coisogenic lines*. Both terms are defined in the first paper of this series (*3*). Coiso-

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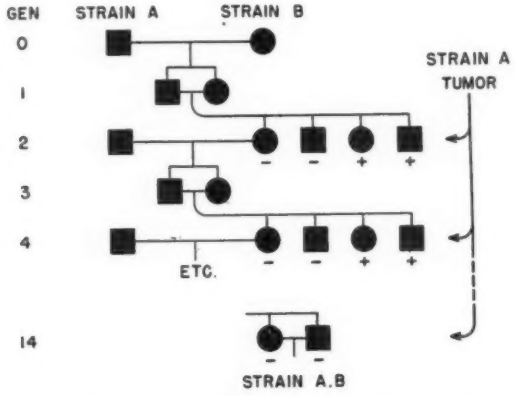
genic lines differ at one locus only or, in special cases, at two loci, and it is convenient to have a name for this locus. We shall call it the *differential locus*. When the differential locus is *H-2*, we shall refer to the IR line as an *H-2* line, where it is *H-3*, as an *H-3* line, etc. The methods of genetic analysis of histocompatibility loci by means of tissue grafts may conveniently be termed *histogenetic methods* to distinguish them from serologic methods such as red cell agglutination.

In 1946, crosses of various mouse strains were set up to produce a group of isogenic resistant lines. These were all lost in the fire that destroyed the Jackson Laboratory in 1947. In January, 1948, about 125 new lines were started from an extensive series of crosses. Many of these were lost due to technical difficulties and others were dropped because of evidence that they represented unnecessary duplications. Twenty-six lines reached the stage where detailed analysis seemed feasible and desirable. We report the results.

Materials and Methods

System of mating.—The system of mating for the production of isogenic resistant lines is shown in text-figure 1. In some cases where difficulty was experienced in obtaining negative animals, departures from the usual system were necessary. While this has some bearing on the extent to which true coisogenicity was achieved, it is not feasible to give the various details.



TEXT-FIGURE 1.—Method for the production of isogenic resistant (IR) lines of mice. In this pedigree chart, A stands for any inbred strain of mice, B for some other strain which may or may not be inbred. Strains A and B are crossed and an F_2 generation raised. This generation and all subsequent even-numbered generations are inoculated with a strain-A tumor. Survivors (indicated by a minus sign) are mated back to strain A. In generation 12, 14, or 16, two survivors are mated together to produce isogenic resistant (IR) strain, A.B. A.B carries some one histocompatibility gene derived from strain B which is different from the corresponding or allelic gene in strain A. Except for this, strain A.B is essentially identical or isogenic with A. Because of this difference at a single histocompatibility locus, grafts made in either direction between A and A.B carry a foreign isoantigen and tend to be rejected.

Designation of IR strains and of generation numbers.—Each IR strain is designated by the symbols, separated by a period, of the two strains used in the original cross. The symbol appearing first is that of the tumor-donating strain with which the IR line is coisogenic. Thus strain LP.RIII comes from the cross LP × RIII and is coisogenic with LP. Where the symbol is unwieldy, abbreviations are used, for example, B10.D2 in place of C57BL/10.DBA/2 and C.B6 in place of BALB/c.C57BL/6.

Since the coisogenicity and homozygosity of an IR line is a function of the number of generations through which it has passed, according to the plan of text-figure 1, and the number of subsequent brother × sister generations, it is useful to have a concise method of designating them. Generations according to the plan of text-figure 1 will be referred to as G generations, the original crossmating being G_0. Subsequent b × s generations will be referred to as F generations, the first b × s mating being F_0 and the offspring of this mating F_1.

Mouse strains.—The following 14 inbred mouse strains were used in the various crosses: A/WySn, AKR/JSn, BALB/cSn, C3H/DiSn, C57BL/6JSn, C57BL/10ScSn, C57L/HeJFeSn, DBA/1JSn, DBA/2WySn, LP/DiSn, NB, P/Sn, RIII/WySn, and ST/JSn. In a few instances the subline was changed during the course of production of the stocks. The subline listed is the subline finally adopted and the one, therefore, that comprises the present genetic background of the IR lines. Eleven noninbred animals or stocks were used as the second parent in various crosses. These (together with some information as to mutant genes which they carried) were: ALB (*ddTt*), BY (*Aa*), CA (*CcCaCaFufu*), CFW (*AABB*), K (*Aabb?CcKiki*), LPK, M (*aaBBCaCa*), SW (*AaBBcc*), T6 (c^{ch} c^{ch}), WA (*AaBbsh-2*), and Y ($A^y a$). Four of these 7 symbols were adopted purely for convenience; they are not official (*4*) and do not represent any stocks now in existence. Stocks CA and WA are still being carried, with somewhat modified genotypes, as multiple gene stocks. SW was a noninbred line of "Swiss" mice. Seventeen inbred and noninbred stocks used in crosses not carried to completion or not analyzed in detail are not included in the above lists. Strain A-*T*, used for linkage tests, is described under "Genetic Methods."

Transplantable tumors.—Most of the tansplantable tumors employed have been described in previous papers (*3*, *5*). Further details in regard to some of them will be found in the excellent summary of Dunham and Stewart (*6*). The history of 4 tumors not previously listed is given in table 1.

At the start of this project, all routine transfers were made subcutaneously on the right flank by trocar (*7*). In 1951 we began changing to the cytosieve method (*8*), and all tumors except for a few that are maintained in the ascites form are now thus carried. This has completely eliminated the problem of infection of extraneous origin (we have had one case of infection in an ascites tumor), and though we suspect that host viruses and perhaps bacterial infections may sometimes localize in transplanted tumors, these are, in our experience, never purulent. Some new

TABLE 1.—*History of tumors, not previously described, which were used in this study*

Designation	Histologic classification of original tumor	Origin				
		Strain	Sex	Date of first transplant	Spontaneous or induced	Method of induction
S840	Rhabdomyosarcoma	A.CA	♀	Jan. 1955	Induced	Methylcholanthrene
S786	Fibrosarcoma	A.SW	♂	Sept. 1952	Induced	Methylcholanthrene
S790	Mammary carcinoma	DBA/1Jax	♀	Oct. 1952	Spontaneous	
S935	Leukemia	AKR.M	♀	Dec. 1956	Spontaneous	

tumors have been transplanted by trocar during the first few transfers before shifting to the cytosieve method. Both methods of implantation were used in the later phase of the production and analysis of IR lines. Further details are given in the text and tables that follow.

Immunization.—The biggest single problem encountered in the production of the IR lines was that, in many of them, all or nearly all the mice of the even-numbered generations, which were inoculated with tumor, succumbed to progressive tumor growth. Either no negatives were obtained or too few for the satisfactory progress of the line. From our present knowledge, it is altogether probable that these lines were segregating for loci other than *H-2*. Where the "strong" *H-2* locus was segregating, somewhere near the expected one fourth (or more) of inoculated animals were usually negative, and maintenance of the line was less difficult.

To overcome this problem we tried, at various times, a change to newer and presumably less virulent tumors, the use of a reduced tumor dose, and immunization prior to tumor implantation with donor-strain blood or with an implant of the tumor in the tail (*9*). All these methods were helpful in some instances but all were open to one or more objections. In particular, the use of a prior tumor implant in the tail led in many tumors to immunization against tumor-specific rather than strain-specific antigens (*see* review in *7*). This was particularly noticeable with methylcholanthrene-induced sarcomas (*10*).

In 1954, at the suggestion of Dr. Avrion Mitchison, we began immunizing with thymus-cell suspensions prepared in the cytosieve. A couple of years earlier, Stoerk *et al.* (*11*) had reported the induction of immunity to a transplantable lymphosarcoma by thymus injections, so that the concept was not new. The latter or, alternatively, immunization with embryonic tissue, has proved by all odds the best method of elevating weak degrees of histoincompatibility between donor and host to a workable level. However, we were not able to exploit it fully until details as to dose, interval, and route were worked out in the experiments described in the first paper of this series (*3*).

The standard procedure which we now use is to give 10 million thymus cells intraperitoneally and the tumor graft subcutaneously 11 days later at a dose that has to be determined individually for each tumor. This method represents a major technical advance.

The linkage test for H-2 *lines.*—The linkage of *H-2* with short-tail or *T* (*12, 13*) provides a convenient method of detecting *H-2* lines. For example, to type strain B10.D2, strain C57BL/10 is crossed to strain A-*T* (*T* on a strain-A background) and the short-tailed F_1 crossed to B10.D2. For brevity, this cross may be written

$$(\text{C57BL/10} \times \text{A-}T) \times \text{B10.D2}.$$

The offspring of this cross are inoculated with a tumor indigenous to strain C57BL/10. Because B10.D2 is coisogenic with C57BL/10, it must carry all genes necessary for the growth of the tumor except for the one differential allele. The cross will segregate for many genes since C57BL/10

and A-*T* are unrelated, but susceptibility and resistance will be determined by the differential locus and by this alone.

Previous investigations have shown strain C57BL/10 to be *H-2*^{b} (*14*). Strain A-*T* is *H-2*^{a}*T*. Strain B10.D2 may 1) be an *H-2* line carrying some allele other than *H-2*^{b}, for example *H-2*^{d}, or it may 2) be *H-2*^{b} (not an *H-2* line). The differential locus must in this latter situation be some other locus, *H-n*. The genotypes of the animals in the final cross and the expected results for these two alternatives then are:

$$1.\ H\text{-}2^b+/H\text{-}2^aT \times H\text{-}2^d+/H\text{-}2^d+.$$

Except for crossovers, which will constitute about 10 percent of the total, normal tailed (+) animals from this cross will be *H-2*^{b}+/*H-2*^{d}+ and hence susceptible to the *H-2*^{b}/*H-2*^{b} tumor; short-tailed mice will be *H-2*^{a}*T*/*H-2*^{d}+ and resistant. Linkage of *T* and resistance will be evident.

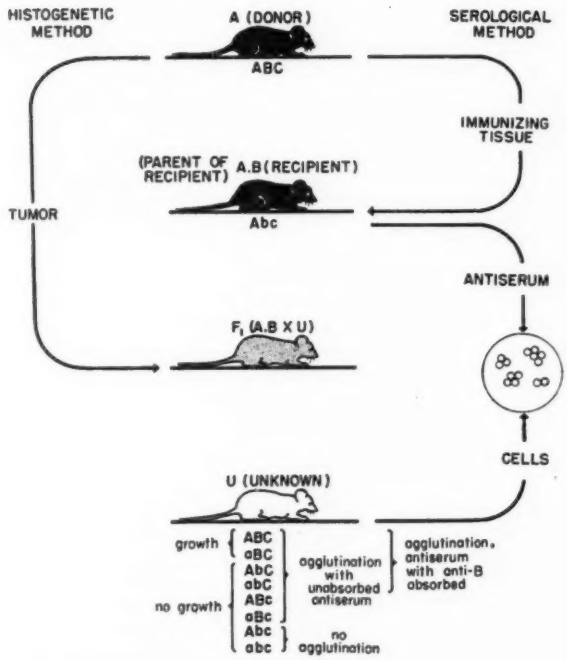
$$2.\ H\text{-}2^b+/H\text{-}2^aT\ H\text{-}n^b/H\text{-}n^a \times H\text{-}2^b+/H\text{-}2^b\ +\ H\text{-}n^d/H\text{-}n^d$$

So far as *H-2* is concerned, all animals will receive *H-2*^{b} from the B10.D2 parent and be susceptible. Animals receiving *H-n*^{a} will be resistant but this will show no linkage with *T*. The occurrence of linkage in this test is thus proof that the IR line is an *H-2* line.

The F_1 test.—Any coisogenic strain pair, A, A.B, with the differential alleles *H-n*^{a} and *H-n*^{b}, respectively, can be used to type any other strain, U, for *H-n*^{a} and *H-n*^{b} (*15*). To type for *H-n*^{a}, the F_1 hybrid produced by crossing A.B × U is inoculated with a tumor from strain A. This F_1 receives from strain A.B *all the strain-A genes* necessary for the growth of the tumor except *H-n*^{a}. If U contributes *H-n*^{a}, the F_1 thereby receives the one missing factor necessary for susceptibility and the tumor will grow; if U does not contribute *H-n*^{a}, the tumor, with the exception of one special situation noted, will not grow. A negative F_1 types U as *not-H-n*^{a}, a positive F_1 types U as probably but not certainly *H-n*^{a}. To type for *H-n*^{b}, A is used as the parent strain and A.B as the tumor donor.

The one exceptional case arises from the fact that some histocompatibility loci determine two or more antigenic components or factors. If the 3 alleles (or pseudoalleles) in strains A, A.B, and U determine, respectively, the components AB, Ab, and aB, the hybrid, Ab/aB, will grow the tumor, AB/AB, even though U and A have different alleles. Strain U, the unknown, does not have to contribute to the F_1 the whole allele present in A, but only the component or components missing from A.B.

This situation is illustrated in text-figure 2. A third component, C, has been added in this figure to contrast the information given by the F_1 test with that given by a strictly serologic test such as red-cell agglutination. The F_1 test in the situation illustrated is a test for the block or group of components BC, the product of one locus or of closely linked



TEXT-FIGURE 2.—A comparison of the results of typing an unknown strain, U, by histogenetic and serologic methods. The coisogenic strain pair, A and A.B, provide the test system. These differ at a single histocompatibility locus. The allele in strain A determines the antigenic components ABC, and that in strain A.B, the components Abc.

loci; the serologic test made with an appropriately absorbed antiserum is a test for the single component C. If made with two absorbed antiserums, anti-A and anti-B, it gives exactly the same information relevant to the identification of alleles as the histogenetic test. Actually, with a complex locus such as *H-2*, a dozen serums each of which had been through several absorptions might be necessary to duplicate the histogenetic information. And even then it would be difficult to be sure that some component had not been missed. Because of the difference in the information which they provide, the two kinds of tests have complemented each other in the analysis of the highly complex *H-2* locus.

For a satisfactory F_1 test, there are certain technical conditions that must be met. In the first place, the lines A and A.B must be adequately coisogenic. Just what constitutes "adequacy" in this instance is not easily determined beyond the obvious point that one wants clear-cut results. We shall defer discussion to a subsequent section.

A second problem concerns the different requirements which are posed through the existence of "strong" and "weak" histocompatibility genes. We have discussed differences in "strength" of histocompatibility genes

elsewhere (*3*) and shall merely cite one example here. Strains C57BL/10, B10.D2, and B10.LP constitute a coisogenic trio; the first two strains differ at *H-2*, and the first and third at *H-3*. C57BL tumor C1498 fails to grow progressively in B10.D2's but does grow in B10.LP's unless these are preimmunized with C57BL/10 tissue. Clearly, the observation that unimmunized hybrids (B10.LP $\times$ U) were susceptible to C1498 would not mean the same thing as the observation that hybrids (B10.D2 $\times$ U) were susceptible. Growth in the first instance would result from the "weak" difference between C57BL and B10.LP and would tell nothing about the contribution of U. To deal with this situation, we have adopted the following rule. When using the F_1 test to type for *H-2* alleles, we use unimmunized F_1 hybrids and give the tumor by trocar. Mice should be at least 7 weeks old. When typing for alleles other than *H-2*, the hybrids are preimmunized and given the tumor as a counted cell suspension according to the methods previously described (*3*). Mice should be at least 10, but preferably 12, weeks old when immunized.

A third technical problem concerns the tumor. Different tumors, or the same tumor at different times, do not necessarily give equivalent results (*3*). With adequate controls (*see* "Control") this problem seldom need be serious.

The component test.—The component test is based on the fact that hybrids of the genotype $H\text{-}2^d/H\text{-}2^k$ are susceptible to strain-A ($H\text{-}2^a/H\text{-}2^a$) tumors 15091a and Sarcoma I (SaI) (*15*). This was explained by the assumption that $H\text{-}2^d$, $H\text{-}2^k$, and $H\text{-}2^a$ determine, respectively, the antigenic components D, K, and DK, an assumption that has since been confirmed by serologic evidence (*16*). The susceptibility of the hybrid may be used to type a strain of unknown *H-2* genotype for $H\text{-}2^d$ or $H\text{-}2^k$. If an unknown is crossed to any one of the known $H\text{-}2^d$ strains (BALB/c, B10.D2, DBA/2), and the F_1 inoculated with SaI, growth indicates that the F_1 is $H\text{-}2^k$. Conversely, if the unknown is crossed to an $H\text{-}2^k$ strain (AKR, C3H, C57BR/a, C57BR/cd), growth of SaI in the F_1 indicates that the unknown is $H\text{-}2^d$. Whether this is a test for $H\text{-}2^d$ and $H\text{-}2^k$, or for the strong D and K antigenic components, will be discussed later.

Controls.—Tumor transplants sometimes grow in resistant animals (false positives), and on rare occasions fail to grow in susceptible animals (false negatives). The degree to which tumors do this probably varies with the transplant generation. The best control for this situation varies with the particular test. A typical control for a test involving hybrids, with C3H.K as one parent and C3H as the tumor donor, consists of 10 C3H.K mice and 10 C3H mice receiving the tumor at the standard dose, plus 10 C3H receiving the tumor at one fifth the standard dose. The C3H.K's provide a control for false positives, the C3H's for false negatives.

In many of the early experiments, separate controls were not run, but routine grafts performed simultaneously with the grafting of an experimental group, for the purpose of propagating the tumor, served as controls for false negatives.

Results

The Isogenic Resistant Lines

Of the 125 lines started, 38 were carried far enough so that tests yielding significant information could be carried out with them; 26 were tested with some thoroughness, and 15 are still maintained.

Identification of H-2 *Lines by Means of the Linkage Test*

Linkage crosses were made (and yielded sufficient animals to warrant discussion) with 23 IR lines. Nine of these crosses are included in table 2. It is apparent that all of them except case #9 show clear linkage. The combined recombination percent, excluding case 9, is 18, a value somewhat larger than the actual crossover percent between *T* and *H-2* (*13*). This is presumably due to the faulty classification of a few animals with respect to both tail and histocompatibility genotype, which usually occurs when classification is based on phenotype alone and not on genetic (progeny) tests.

Case 9 was the test of the coisogenic strain pair C3H and C3H.K. It gave 33 recombinants out of a total of 53 mice, or 62 percent. There is thus no evidence of linkage, and we may conclude tentatively that this is not an *H-2* line, though the large excess of animals that succumbed to the tumor (41 out of 53) despite preimmunization by inoculations in the

TABLE 2.—*Results of the linkage test for the detection of* H-2 *lines*

Case No.	Cross	Tumor and method of inoculation*	Numbers of Mice†				Recombination percent
			Normal tail		Short tail		
			+	−	+	−	
1	(A × A-*H-2*^{b}*T*) × A.SW	15091a-T	22	3	7	12	23
2	(AKR × A-*T*) × AKR.K	Wagner-T	17	3	1	16	11
3	(C57BL/6 × A-*T*) × B6.D2	C1498-T	12	5	2	16	20
4	(C57BL/10 × A-*T*) × B10.D2	S653-TT	12	6	0	13	19
5	(C3H × A-*T*) × C3H.NB	6C3HED-S	14	3	4	11	22
6	(DBA/1 × A-*T*) × D1.ST	S790-T	19	2	3	21	11
7	(P × A-*T*) × P.CFW	S637-T	17	4	1	15	14
8	(LP × A-*T*) × LP.B10	D22-S	33	5	10	17	23
9	(C3H × A-*T*) × C3H.K	6C3HED-TT	16	8	25	4	62

*The tumor is always indigenous to the first of the 3 strains listed under "cross." The method of inoculation is indicated after each tumor.

T = inoculation by trocar; TT = inoculation in the tail followed by subcutaneous inoculation by trocar; S = inoculation subcutaneously with a cell suspension prepared by sieve.

†Age of mice at tumor inoculation ranged from 28 to 144 days. All mice in columns headed + succumbed to the tumor; mice in columns headed − survived. The two middle columns are the recombination classes.

tail makes the data somewhat unsatisfactory. We have already described C3H.K as an *H-1* line (*17*) and shall present additional evidence in a subsequent section of this paper.

Besides the 9 lines included in table 2, 3 lines (B6.M, D1.LP, and D2.WA) which are included in subsequent tables dealing with additional tests, and 8 lines which were dropped without further testing, were shown by linkage of resistance with *T* to be *H-2* lines. Three lines besides C3H.K gave no evidence of linkage. With one of them, AKR.RIII, the numbers were inadequate for a satisfactory test. With another, C.AKR, the tumor employed, S621, has been shown to be capable of evoking immunity in the native BALB/c strain (*18*, *19*), which leaves the results somewhat open to question. The evidence for absence of linkage in the third, C3H.DS, was quite clear, and an F_1 test with C3H.K gave no deaths out of 7 animals inoculated, which indicates that it, like C3H.K, was an *H-1* line. All 3 lines were subsequently dropped or lost.

Thus of 23 lines subjected to the linkage test, 19 were found to be *H-2*, 2 were *H-1*, and 2 others were possibly, but not surely, *not-H-2*.

Typing IR Lines for Various H-2 *Alleles by Means of the F_1 Test*

The inbred members of the coisogenic pairs shown in table 2, cases 1 through 7, together with the *H-2* allele that each carries (*5*) are: A, $H\text{-}2^a$; AKR, $H\text{-}2^k$; C57BL/6 and C57BL/10, $H\text{-}2^b$; C3H, $H\text{-}2^k$; DBA/1, $H\text{-}2^q$; and P, $H\text{-}2^p$. Isogenic resistant strain B10.D2 was shown by the component test to be $H\text{-}2^d$, as is expected, since the histocompatibility locus foreign to C57BL/10 which it carries came from the $H\text{-}2^d$ line, DBA/2. These coisogenic strain pairs, used in the F_1 test, thus provide systems for typing any unknown line for $H\text{-}2^a$, $H\text{-}2^b$, $H\text{-}2^k$ (two distinct test systems), $H\text{-}2^p$ and $H\text{-}2^q$. For reasons that will subsequently become apparent, strain pairs A.SW and A, with A.SW as the tumor donor, A.CA and A, with A.CA as the tumor donor, AKR.M and AKR, with AKR.M as the tumor donor, and RIII and RIII.P, with RIII as the tumor donor, were also adopted as test systems. All these together make a total of 11 typing systems (the pairs C57BL/6 and B6.D2, and C57BL/10 and B10.D2, were employed with the same tumor, C1498, and are treated as one test system).

Tables 3 and 6 show the results of the F_1 test with these 11 systems applied to 26 IR lines. There are 126 F_1 test groups with 3 to 51 (usually 10 to 20) mice per group. As indicated under "Methods," growth of the transplant shows that the unknown probably carries the *H-2* allele of the tumor donor; failure to grow shows that it does not carry this allele. It must be remembered that most widely used transplantable tumors, when tested in a variety of foreign strains, will grow in occasional animals of at least some of the strains (*7*, *20*). These "false positives" do not indicate genetic susceptibility. Frequently survival time of the animals that die is prolonged as compared with grafts in the native strain (*3*). While the occurrence of a few deaths does not indicate susceptibility, the occurrence of any survivors almost unfailingly indicates resistance,

especially if simultaneous isograft controls (or routine grafts for the purpose of propagating the tumors) are all positive.

The first point to consider is whether the test animals fall into clearly susceptible and resistant categories. Of the 126 test groups, there were no deaths in 74; 100 percent deaths in 18. There is no reasonable question about the interpretation in these cases. It will be convenient to consider the remaining 34 cases by the allele under test, taking the vertical columns of table 3 more or less in order.

The test for *H-2*^{a} was done before we had established the rule that all *H-2* tests be done by the trocar method; SaI was given as a cell suspension, 625,000 cells per mouse. Most of the groups were unambiguous, no deaths occurring. In 4 groups totaling 56 mice, 6 mice succumbed, deaths occurring 62 to 83 days after tumor inoculation. In one group (testing AKR.K), 13 out of 14 mice succumbed, death occurring 27 to 62 days after tumor inoculation. The test with AKR.K was repeated with 24 additional F_1 mice, SaI being given by trocar. All succumbed. Evidently this F_1 hybrid group should be classed as susceptible, and AKR.K is therefore *H-2*^{a}.

The tests for *H-2*^{b} with tumor C1498 were mostly unambiguous, though there were 6 partly susceptible groups with 6 to 63 percent deaths. Every death in these groups, however, occurred later than the 151 deaths in the 10 susceptible (100 percent death) groups, so that if long survival is allowed as a criterion of resistance, all ambiguity is removed. We have seen C1498 increase in virulence (capacity to grow in foreign strains) over the long period that we have worked with it (*20*), and it has recently become so virulent that it has lost much of its value for genetic studies. The tests for *H-2*^{b} were done in 3 separate experiments; 23 out of the 29 deaths in resistant groups occurred in the last experiment.

The tests for *H-2*^{d} (tumor S842) gave 2 cases with 100 percent deaths, 2 with no deaths, and 5 with 8 to 59 percent deaths. The highest value was given by the F_1 (C57BL/10 $\times$ D1.C). This appears to represent a special case that we shall subsequently discuss further. Except for this one case, the mice that died in the resistant groups (groups with survivors) tended to live longer than the mice in the susceptible groups, but the separation was not nearly as sharp as for C1498. This is one of the less satisfactory tests, but even so, there seems to be little doubt as to the correct interpretation of the results. The test with B10.SW, with 100 percent deaths, included only 6 mice, but we give additional evidence for this in a later table.

The tests for *H-2*^{f}, *H-2*^{k} (*see* table 6 as well as table 3), *H-2*^{p}, and *H-2*^{s} gave 4 cases with 100 percent deaths, 42 with no deaths, and 5 with 6, 7, 8, 9, and 13 percent deaths, respectively. There can be little question that the last 5 belong with the resistant groups.

The tests for *H-2*^{m}, with AKR.M as the tumor-donor, gave no deaths in 3 cases, 5 percent deaths in 1 additional case. These require no comment. There were 38 percent deaths among the F_1 (AKR $\times$ AKR.ALB), but 11 AKR.ALB controls implanted simultaneously gave 55 percent

TABLE 3.—*Results of typing 26 IR lines for 10 different* H-2 *alleles—figures show percent deaths from tumor transplants (numbers of mice in parentheses)**

Indicated *H-2* type of unknown parent	Case No.	Unknown parent	Coisogenic test pair. The four headings show: *H-2* allele of tumor donor, tumor donor, tumor, known parent									
			H-2^a A SaI † A.SW	*H-2*^{b} C57BL C1498 ‡ B10.D2	*H-2*^{d} B10.D2 S842 C57BL	*H-2*^{f} A.CA S840 A	*H-2*^{k} AKR S775 AKR.K	*H-2*^{m} AKR.M S935 § AKR	*H-2*^{p} P S637 P.CFW	*H-2*^{q} DBA/1 S790 D1.ST	*H-2*^{r} RIII S674 RIII.P	*H-2*^{s} A.SW S786 A
H-2^a	1	AKR.K	93(14)	0(13)‖	.....	.....	.....	.....	0(13)	.....	.....	.....
H-2^b and *H-2*$^{b'}$?	2	A.BY	0(6)	100(15)	.....	.....	.....	.....	.....	.....	.....	13(8)
	3	B10.BY	.....	100(20)	20(10)	0(9)	0(5)	.....	.....	.....	.....	.....
	4	B10.LP	.....	100(14)	.....	.....	.....	.....	.....	.....	.....	7(15)
	5	C.B6	0(17)	100(26)	.....	.....	0(7)	.....	.....	.....	.....	0(15)
	6	C3H.LPK	18(17)	100(14)	.....	.....	0(10)	.....	.....	.....	.....	0(13)
	7	C3H.SW	.....	100(16)	.....	0(17)	0(13)	.....	.....	.....	20(10)	.....
	8	D1.LP	0(17)	100(16)	.....	.....	0(16)	.....	.....	.....	.....	0(14)
	9	D2.WA	10(10)	100(16)	.....	.....	.....	.....	.....	.....	.....	.....
	10	L.WA	0(6)	100(14)	.....	.....	.....	.....	.....	.....	.....	.....
	11	P.CFW	.....	100(10)¶	.....	.....	.....	.....	.....	.....	.....	.....
H-2^c	12	D1.C	0(9)	0(17)	59(51)	0(17)	0(9)	88(17)	.....	43(28)	65(17)	0(17)
H-2^d	13	B10.D2	.....	.....	+	.....	.....	.....	.....	.....	.....	0(18)
	14	B10.SW	0(16)	0(16)	100(6)	0(17)	0(16)	.....	.....	.....	.....	0(12)
	15	ST.T6	0(14)	19(16)	100(17)	0(17)	7(15)	.....	.....	.....	.....	0(20)
H-2^f	16	A.CA	0(11)	6(16)	54(13)	+	9(11)	.....	0(16)	0(17)	41(22)	0(10)
	17	B6.M	0(13)	30(20)	8(13)	100(17)	0(16)	.....	.....	.....	.....	0(20)
H-2^m	18	AKR.M	0(16)	0(17)	18(11)	0(17)	6(18)	+	0(8)	0(17)	50(12)	0(21)
H-2^k	19	AKR.ALB	0(17)	0(15)	.....	.....	100(13)	38(13)	.....	.....	.....	0(10)
	20	C3H.K	.....	.....	.....	.....	100(12)	5(20)	.....	.....	.....	0(18)
	21	D1.ST	8(13)	63(19)	.....	0(17)	100(15)	.....	.....	.....	.....	0(14)
H-2^p?	22	RIII.P	0(16)	11(9)	0(18)	0(14)	.....	.....	.....	0(11)	.....	0(16)

H-2^q	23	B6.Y	0(13)	20(20)	.....	0(14)	0(11)	.....	.....	100(17)	0(3)	.....
H-2^r	24	LP.RIII	6(16)	0(17)	.....	.....	.....	0(7)	.....	0(10)	84(31)	.....
H-2^s	25	A.SW	.....	0(17)	43(14)	0(17)	0(17)	.....	0(16)	0(13)	80(49)	+
H-2^t	26	C3H.NB	0(15)	0(15)	0(18)	0(16)	0(14)	0(10)	.....	7(15)	88(17)	0(18)

*All tumors given subcutaneously by trocar unless otherwise noted. Age of mice at tumor inoculation, 7 to 10 weeks, with rare exceptions.

†SaI was given subcutaneously in the form of a cell suspension, 625,000 cells per mouse, instead of by trocar. This probably accounts for the one survivor in the test of strain AKR.K. In a repetition of this test in which the tumor was given by trocar, there were 24 deaths in 24 mice.

‡The 151 mice in the *H-2*^{b} group which were inoculated with C1498 succumbed in 12 to 21 days. The 27 mice from *non-H-2*^{b} groups, which succumbed, out of 227 inoculated, survived 27 to 83 days. This long survival is interpreted as an indication of genetic resistance.

§ The 21 mice in groups *H-2*^{a} and *H-2*^{b} which succumbed to S935 survived for 21 to 35 days. Nine AKR.M which received implants at the same time died within 14 days.

‖ Known parent was B6.D2 instead of B10.D2.

¶ Mice from the cross B10.D2 ×P.CFW were inoculated with S653 instead of C1498. Six of the ten mice received an inoculation in the tail followed by a subcutaneous inoculation by trocar; the remainder received the tumor subcutaneously as a cell suspension, 125,000 cells per mouse.

deaths, and 6 AKR's, no deaths, so the partial susceptibility would appear to be due to the AKR.ALB parent. We shall present evidence in a future publication that the histocompatibility difference between AKR.ALB and its coisogenic partners is a weak one. We interpret this as a negative group. There were 15 deaths out of 17 mice (88 percent) among the F_1 (AKR $\times$ D1.C), though the survival time was prolonged as compared with that of the pure strain AKR. We shall discuss this unusual case later.

The tests for *H-2*^{q} are clear except that the F_1 (D1.ST $\times$ D1.C) gave 12 deaths in 28 mice. Since these mice are on a homozygous DBA/1 background, the partial susceptibility in this instance may be due to the low resistance of the DBA/1 strain to tumor grafts in general (*20, 21*). D1.C is probably *not-H-2*^{q}.

By all odds the least satisfactory results were given by the tests for *H-2*^{r}, the allele for which RIII/Wy is the type strain. The data for *H-2*^{r} in table 3 are the combined results of 6 tests. These, plus an added test with strain LP, are shown separately in table 4. Most instructive are cases 8 and 9, which test strains LP and LP.RIII. Strain LP is known from previous work to be *H-2*^{b}, (*5, 15*); strain LP.RIII is *not-H-2*^{b} (table 3, case 24). The F_1 (RIII.P $\times$ LP) implanted with S674 (RIII origin) gave 0 deaths in 19 mice (case 8, table 4), whereas the F_1 (RIII.P $\times$ LP.RIII) gave 10 deaths in 15 mice in tests run simultaneously with those in case 8, 16 deaths in 16 mice in tests run subsequently (case 9). The occurrence of survivors in case 9 makes these tests unsatisfactory; nevertheless, they do indicate that LP.RIII carries, and LP lacks, some factor necessary for the growth of the RIII tumor. Since LP has been proved to be *H-2*^{b} and LP.RIII *not-H-2*^{b}, and since they presumably have no difference other than this, LP.RIII must carry *H-2*^{r} or an allele related to *H-2*^{r}. This is of course expected since the allele came from RIII/Wy.

It follows also that the difference between RIII and RIII.P must be an *H-2* difference. If it were not an *H-2* difference, the crosses to LP and LP.RIII, which differ only at *H-2*, should have given identical results. The occurrence of survivors in case 9 probably indicates that the *H-2* difference is not the only difference between RIII and RIII.P; in other words, that these strains are not coisogenic. We will discuss this further.

An added difficulty with the test for *H-2*^{r} was that tumor S674 became so virulent sometime during 1956 that the majority of mice in all hybrid groups died in the tests run during and after November of that year. (This also made it impossible to carry out a satisfactory linkage test similar to the ones shown in table 2.) Nevertheless, only LP.RIII brought in the missing factor necessary for complete susceptibility. The most probable conclusion seems to be that LP.RIII is *H-2*^{r}, and all other strains included in table 4, *not-H-2*^{r}.

We may now consider the positive tests in table 3, that is, the tests giving 100 percent deaths. With them, for the reasons given, we include the test of AKR.K for *H-2*^{a} and the test of LP.RIII for *H-2*^{r}. Seventeen

TABLE 4.—*Results of typing for* H-2^r*—figures show fraction mice dying in tests carried out on different dates*

Case No.	Cross	Date of tumor inoculation*					
		2/24/55	3/16/55	1/26/56	11/29/56	1/4/57	4/8/57
1	RIII.P × A.CA			0/4			9/18
2	× A.SW			0/5	16/17		23/27
3	× AKR.M			0/2		6/10	
4	× B6.Y			0/3			
5	× C3H.NB						5/17
6	× C3H.SW			2/10			
7	× D1.C						11/17
8	× LP	0/6	0/13				
9	× LP.RIII	8/10	2/5	6/6		10/10	

*RIII tumor S674 given by trocar on all dates except 2/24/55 when a cell suspension was used, 3,125,000 cells per mouse.

of the 26 IR lines in table 3 responded positively. Four others, B10.D2, A.CA, AKR.M, and A.SW, appear in the table as both tumor donors and as unknowns, and would undoubtedly have shown a positive response in the square where a + has been inserted had the test been performed.

Two tests of self-consistency, or consistency with the results of earlier histogenetic studies, may be applied to these results. No line shows 100 percent growth in more than one column of the table. Such a result would indicate that the line was homozygous for two distinct *H-2* alleles, which is impossible. In nearly every case where we have the information to form an expectation, the IR line appears in the expected *H-2* allelic group. Thus B10.LP and D1.LP both test as *H-2^b*. This is expected since LP is *H-2^b* (*5*, *15*). Similarly C.B6 (BALB/c.C57BL/6) turns out to be *H-2^b*; B10.D2, *H-2^d*; and D1.ST, *H-2^k*. We do not know the allele characteristic of the BY and WA strains, but it is noteworthy that strains A.BY, B10.BY, D2.WA, and L.WA all reacted as *H-2^b* (or *H-2$^{b'}$*, *see* later). On the other hand, C3H.SW reacted as *H-2^b*, B10.SW as *H-2^d*, and A.SW as *H-2^s*. Also AKR.K and C3H.K turned out to be *H-2^a* and *H-2^k*, respectively, and B6.M and AKR.M, *H-2^f* and *H-2^m*. This is not surprising, since SW, K, and M were noninbred stocks. SW, a "Swiss" stock, was maintained in quantity and might well have carried a diverse assortment of genes.

Two valid exceptions are line LP.B10, which reacted in the linkage test for *H-2* lines as if distinct from LP at the *H-2* locus (table 2, case 8), though LP and C57BL/10 have both been described as *H-2^b*, and line D1.C (DBA/1.BALB/c), which reacted in the F_1 test as *not-H-2^d* (table 3, case 12), though BALB/c is *H-2^d*. These will be discussed further.

There are 5 cases (# 3, 4, 10, 19, and 20) in which the positive outcome of a test in table 3 has special significance. Line B10.BY tested as *H-2^b*. But C57BL/10, its coisogenic partner, is also *H-2^b*. These data, as they stand, permit two interpretations. Either B10.BY actually carries some allele, *H-2$^{b'}$*, not identical with *H-2^b*, but so similar that the test recorded in table 3 did not reveal the difference, or else it *is* *H-2^b*, and hence must

differ from C57BL/10 at some locus other than *H-2*. The other 4 coisogenic pairs to which this same reasoning applies are C57BL/10 and B10.LP, C57L and L.WA, AKR and AKR.ALB, and C3H and C3H.K. These will be discussed further.

We now turn to certain tests in table 3 where a negative outcome has special significance. We may start with case 6, the test in which 10 mice, (AKR.K × C3H.LPK)F_1, were inoculated with AKR tumor S775 and all survived. This is a test for $H\text{-}2^k$; the negative result shows C3H.LPK to be *not-H-2*^{k}. Strain C3H is known from earlier work to be $H\text{-}2^k$ (certain qualifications in regard to the distinction between $H\text{-}2^k$ and $H\text{-}2^{k'}$ are discussed later). Since C3H is $H\text{-}2^k$ and its coisogenic mate *not-H-2*^{k}, we may conclude that C3H.LPK differs from C3H at the *H-2* locus and is an *H-2* line. This is confirmed by the test for $H\text{-}2^b$ (case 6) whose positive outcome showed C3H.LPK to be $H\text{-}2^b$. Seven other lines doubly established as *H-2* lines are A.BY (case 2), C3H.SW (case 7), B10.SW (case 14), ST.T6 (case 15), B6.M (case 17) (also proved to be an *H-2* line by the linkage test), B6.Y (case 23), and LP.RIII (case 24) (with qualifications in this last test noted earlier).

Four additional IR lines were shown by the same negative test already cited to lack the *H-2* allele present in their inbred coisogenic mate, and hence to be *H-2* lines, but gave no positive F_1 test that could establish them as belonging in a known *H-2* group. These were D1.C (case 12), A.CA (case 16), AKR.M (case 18), and C3H.NB (case 26). We may include with this group, line A.SW, which was shown by the linkage test to be an *H-2* line (table 2, case 1) but which was not identified as carrying any known *H-2* allele by the F_1 tests in table 3 (case 25). Since these 5 lines were not shown by the F_1 test to carry any known *H-2* allele, we need to examine the possibility that they carry some new *H-2* allele. We shall draw on both the data from the F_1 test in table 3 and the component test in table 5.

Line A.SW

This line was shown by the linkage test (table 2, case 1) to carry some *H-2* allele other than that in strain A. It is not $H\text{-}2^a$. It was typed by the F_1 test for, and shown to lack, $H\text{-}2^b$, $H\text{-}2^d$, $H\text{-}2^f$, $H\text{-}2^k$, $H\text{-}2^p$, $H\text{-}2^q$, and $H\text{-}2^r$ (table 3, case 25; table 4). These are all the alleles previously identified by histogenetic methods (*5*, *22*; *see also* next paragraph for $H\text{-}2^f$). The component test (table 5, case 14) shows A.SW to lack K. While no histogenetic tests with A.SW have been reported previously, Hoecker, Counce, and Smith (*16*) have already shown by the red-cell agglutination test that the combination of *H-2* components in A.SW differs from that in 6 of the 8 *H-2* alleles listed. Drawing partly on our unpublished data, they assigned the allele in A.SW the symbol $H\text{-}2^s$. Its identity as a new allele has been substantiated by all subsequent tests.

Line A.CA

This was typed by the F_1 test for, and shown to lack, $H\text{-}2^a$, $H\text{-}2^b$, $H\text{-}2^d$, $H\text{-}2^k$, $H\text{-}2^p$, $H\text{-}2^q$, $H\text{-}2^r$, and $H\text{-}2^s$ (table 2, case 16). Allen (*22*) has

TABLE 5.—*Results of the component test*

Case No.	Tumor*	Cross	Type of cross	Fraction dying
1	SaI	AKR	Control	0/16
2	SaI	B10.D2		0/10
3	SaI	D1.C		2/8
4	15091a	B10.D2 × BALB/c	$H\text{-}2^d \times H\text{-}2^d$ (control)	1/24
5	SaI	B10.D2 × AKR	$H\text{-}2^d \times H\text{-}2^k$ (control)	13/13
6	SaI	B10.D2 × C3H		15/15
7	15091a	B10.D2 × C3H		8/8
8	SaI	B10.D2 × D1.ST		15/15
9	SaI	B10.D2 × C3H.SW	$H\text{-}2^d \times H\text{-}2^b$ (not K)	5/16
10	SaI	B10.D2 × LP	$H\text{-}2^d \times H\text{-}2^b$ (not K)	3/20
11	15091a	BALB/c × B6.M	$H\text{-}2^d \times H\text{-}2^f$ (not K)	0/14
12	SaI	B10.D2 × LP.RIII	$H\text{-}2^d \times H\text{-}2^r$ (not K)	2/18
13	SaI	B10.D2 × RIII	$H\text{-}2^d \times H\text{-}2^r$ (not K)	0/18
14	SaI	B10.D2 × A.SW	$H\text{-}2^d \times H\text{-}2^s$ (not K)	0/20
15	SaI	B10.D2 × C3H.NB	$H\text{-}2^d \times H\text{-}2^q$ (not K)	3/17
16	SaI	B10.D2 × AKR.M	$H\text{-}2^d \times H\text{-}2^m$ (K)	17/17
17	SaI	DBA/2 × AKR.M		10/11
18	15091a	C57BR/a × A.CA	$H\text{-}2^k \times H\text{-}2^f$ (not D)	1/8
19	15091a	C3H × B6.M	$H\text{-}2^k \times H\text{-}2^f$ (not D)	0/18
20	15091a	C57BR/a × AKR.M	$H\text{-}2^k \times H\text{-}2^m$ (not D)	0/9
21	15091a	C3H × C3H.NB	$H\text{-}2^k \times H\text{-}2^q$ (not D)	0/9
22	SaI	AKR × D1.C	$H\text{-}2^k \times H\text{-}2^s$ (D)	17/17

*Tumors SaI and 15091a are native to strain A. This strain carries the allele $H\text{-}2^a$ with both D and K components. Mice were 7 to 10 weeks of age at the time of tumor inoculation. All tumor inoculations performed by trocar.

previously described an allele, *H-2f*, present in stock F^h, which she showed to be distinct from all the alleles listed except *H-2s*, which at that time was not known. Stock F^h is now lost. However, stock F^h carried in association with *H-2f*, and stock A.CA now carries and is homozygous for, the gene *Fu*, derived in both cases from stock CA. Gene *Fu* is closely linked with *H-2* (*13*). It seems likely, therefore, that the *H-2* allele in these 3 stocks is the same. In any case, since stock F^h is lost, there can be no conflict in assigning the allele in A.CA the symbol *H-2f*.

Line AKR.M

It was shown by the F_1 test that the *H-2* allele in AKR.M is not *H-2a*, *H-2b*, *H-2d*, *H-2f*, *H-2k*, *H-2p*, *H-2q*, *H-2r*, or *H-2s* (table 3, case 18; table 4, case 3). The test for *H-2r*, like others for this allele, left something to be desired, but two additional tests rule out the possibility that AKR.M is *H-2r*. Seven mice from the (AKR × RIII)F_1 were inoculated with AKR.M tumor S935 and all survived, and identical results were obtained with LP.RIII (data not included in table). This excludes the possibility that RIII and AKR.M carry the same *H-2* allele. Strains RIII and AKR.M were also tested for K by the component method, the outcome with RIII being negative, with AKR.M, except for 1 survivor in 28 mice,

positive (table 5, cases 13, 16, and 17). As we point out, this may not prove that RIII lacks K, in fact serologic tests have indicated that RIII carries K (*23*), but it does indicate a difference from AKR.M. A test for D was negative (table 5, case 20).

Our usual test system for *H-2*^{k} is the coisogenic pair AKR and AKR.K. As a further check, AKR.M was tested also with the pair C3H–C3H.NB, again reacting as *not-H-2*^{k} (table 6).

TABLE 6.—*Evidence that AKR is, and AKR.M is not,* H-2^k

Host	Tumor donor	Tumor	Percent deaths (No. of mice in parentheses)
C3H.NB (control)	C3H (*H-2*^{k})	E9514	0 (9)
AKR (control)	"	"	0 (10)
C3H.NB × AKR	"	"	100 (5)
C3H.NB × AKR.M	"	"	0 (15)

The results of this test are so definite that AKR.M can hardly be merely a minor variant of the *H-2*^{k} group. Nevertheless, since minor variants were indicated in an earlier study, it seemed desirable to test AKR.M against some other *H-2*^{k} (or *H-2*$^{k'}$) lines. The (AKR × BR/cd) F_1, inoculated with S935 gave 0 deaths out of 12 mice, and the (AKR × ST)F_1 2 out of 20, confirming the strength of the difference between AKR.M and known *H-2*^{k} (or *H-2*$^{k'}$) lines. A test of AKR.M against C57BR/a, the type line for the presumed variant *H-2*^{k} allele, would have been desirable but was not made.

We conclude that AKR.M carries a new *H-2* allele, which we may call *H-2*^{m}.

Line D1.C

This line was tested for, and shown to lack, *H-2*^{a}, *H-2*^{b}, *H-2*^{d}, *H-2*^{f}, *H-2*^{k}, *H-2*^{m}, *H-2*^{q}, *H-2*^{r}, and *H-2*^{s} (table 3, case 12). The test for *H-2*^{m} was somewhat ambiguous since there were 88 percent deaths (though all the mice that died showed long survival), but D1.C tested by the component test reacted as D, whereas AKR.M tested as K, not-D (table 5, cases 22, 16, 17, and 20). Line D1.C was not tested for *H-2*^{p}, but Hoecker, Counce, and Smith (*16*) have shown *H-2*^{p} to lack D. A clearer test against *H-2*^{r} would be desirable (or a test showing RIII to lack D). So also would be a test against YBL/Rr, which an earlier study indicated as the bearer of a minor variant of *H-2*^{d} and which we called *H-2*$^{d'}$. We assign the allele in D1.C the symbol *H-2*^{c}. Should it be shown later to be identical with *H-2*$^{d'}$, the symbol *H-2*^{c} should probably be retained since it is useful to have an IR line as the type line for any allele.

We have already pointed out that since the allele in D1.C was derived from strain BALB/c, there is a presumption that it should be *H-2*^{d}. The high percent of deaths in the F_1 test for *H-2*^{d} (table 3, case 12) does in fact suggest some similarity to *H-2*^{d}. Also it shares the D component with

H-2^{d}, and this component is lacking in DBA/1. Since crossovers between components of the *H-2* system are known to occur (*13*, *24*), it may plausibly be assumed that *H-2*^{c} was derived by a crossover between the components of *H-2*^{q} and *H-2*^{d} during the synthesis of line D1.C. If so, serologic tests should reveal no components foreign to these two alleles.

Line C3H.NB

This line was subjected to all tests necessary to establish it as probably a new allele except the test for *H-2*^{p} (the test for *H-2*^{r} was particularly poor) (table 3, case 26; table 4, case 5; table 5, case 21). Unfortunately our test stock for *H-2*^{p}, P.CFW, which carried multiple recessive genes and was a difficult breeder, was lost before the missing test was made.

Interpretation of the Component Test

The component test is based on the fact that hybrids of the genotype *H-2*^{d}/*H-2*^{k} will grow strain A (*H-2*^{a}/*H-2*^{a}) tumors SaI and 15091a (*see*, for example, table 5, cases 5, 6, 7, and 8). We now find that the hybrids *H-2*^{m}/*H-2*^{d} (table 5, cases 16 and 17) and *H-2*^{k}/*H-2*^{c} (table 5, case 22) also grow these tumors (though there was one survivor in the former case). This suggests that the test is indicative of specific components or combinations of components rather than of alleles.

The demonstration by Gorer and others (*13*, *24*) of recombination within the *H-2* locus permits an interesting explanation of the component effect. If *H-2*^{a} were derived from a recombination occurring in an *H-2*^{d}/*H-2*^{k} hybrid, its *H-2* antigen would, of necessity, be compatible with the hybrid. We know (*see* review in the final section of this paper) that *H-2*^{a}, *H-2*^{d}, and *H-2*^{k} carry, respectively, the antigenic components DFMEK, DFME^{a}–, and —EK (components common to all 3 alleles omitted). These structures are compatible with the recombination hypothesis. Whether all the components, DFMEK and perhaps also C and H and others as yet undetected, present in *H-2*^{a} have to be present in a hybrid for the growth of *H-2*^{a} tumors is uncertain. For the purposes to which we have put the component test, we may postulate with considerable confidence that growth in the hybrid proves the presence of the "strong" D and K components (or of components closely related to these). However, failure to grow does not necessarily prove their absence since this may be due to the absence in the hybrid of other components such as F, M, or E. Strain RIII/Wy, which types as K by the agglutination test (*23*), but not-K by the component test, is probably a case in point.

Interpretation of the F_1 Test

In the sections on the F_1 test under "Materials and Methods," we pointed out that this test identifies a group of antigenic components which may not, however, comprise the total component system determined by any *H-2* allele. This situation was illustrated in text-figure 2. The 10 IR lines which gave 100 percent growth when tested against the coisogenic pair C57BL/10–B10.D2 (table 3, cases 2–11) must have a number of

antigenic components in common with the C57BL/10 allele, *H-2*^{b}, but are not necessarily identical. What is the likelihood that they are, in fact, different?

In an earlier study (*5*) we typed 22 inbred strains for *H-2* by the use of a linkage method that is subject to exactly the same qualifications as the F_1 test. A number of these lines have been used subsequently in serologic studies of the *H-2* system (*16*, *23–30*), and in no case in which we found alleles to be similar have serologic tests shown them to be different. It may be that this consistency is due in some cases to the fact that certain inbred strains are closely related and hence have received the same histocompatibility allele from a common ancestor. Experience would seem to indicate that the grouping of strains in table 3 according to the allele which they carry is likely to prove accurate in its essentials, although it remains possible that a large group like that included under *H-2*^{b} will later turn out to have significant subdivisions. There is in fact some evidence that this is true.

Minor H-2 *Differences*

Snell, Smith, and Gabrielson (*5*) reported minor variants of *H-2*^{d} and *H-2*^{k}, which were designated *H-2*$^{d'}$ and *H-2*$^{k'}$, respectively. *H-2*$^{d'}$ was found in YBL/Rr. Serologic tests have likewise shown a minor difference between YBR/Wi (related to YBL/Rr) and other *H-2*^{d} lines (*27*). We know of no additional evidence either refuting or confirming the slight differences in the *H-2*^{k} group.

Such differences are, by their very nature, difficult to prove. We have turned up additional indications of minor variants in this study but the evidence is mostly inconclusive. Isogenic resistant strain LP.B10 came from a cross of two *H-2*^{b} lines so that both members of the coisogenic pair LP and LP.B10 should carry this allele. Yet the linkage test (table 2, case 8) showed an *H-2* difference. The IR line was lost in the last G generation due to failure to get any negatives, a situation suggestive of a weak difference. Coisogenic lines C57L and L.WA have both tested *H-2*^{b}. Yet a test for linkage with *T* (data not given) showed a deficiency of recombination classes, though the large excess of deaths made the results rather unsatisfactory. If L.WA was actually a minor variant of *H-2*^{b}, then D2.WA presumably was also one. Both lines have been dropped.

Histocompatibility-1

The coisogenic strain pair C3H and C3H.K is unusual among the 26 pairs included in table 3 since the two members are visibly different. Strain C3H has a colored coat (wild type), strain C3H.K is albino. A tabulation of data from the generations receiving tumor transplants during the production of the IR line showed a significant association of albinism and resistance. Since albinism is in linkage group I, whereas *T* and *H-2* are in linkage group IX, we were able to conclude that C3H and C3H.K

differ at some new locus, which we called histocompatibility-1 or *H-1* (*17*). The alleles in C3H and C3H.K were assigned the symbols *H-1*^{a} and *H-1*^{b}, respectively. We here present a variety of evidence confirming the existence of *H-1*.

As already pointed out, the C3H–C3H.K coisogenic strain pair was one of very few that failed to show association of *T* and resistance in the linkage test for *H-2* lines (table 2, case 9). This pair must be differentiated by some locus other than *H-2*.

Strain C3H is H-2^k. The results of the F_1 test (table 3, case 20) indicate that C3H.K is also *H-2*^{k}. This is further evidence that the difference is not at *H-2*. However, since the histocompatibility difference between C3H and C3H.K is a weak one, and the C3H tumor E9515 used in the test kills about 50 to 75 percent of pure strain C3H.K (*3*), the data in table 3 are not conclusive. Table 7 shows the results of a refinement of the F_1 test. C3H.NB is an *H-2*^{b} line coisogenic with both C3H and C3H.K. Mice from the usual type of cross, (C3H.K $\times$ C3H.NB)F_1, were preimmunized with C3H embryo and then given E9514 as a cell suspension, 125,000 cells per mouse. All were dead within 21 days, though under these conditions all C3H.K controls were alive at 21 days and only 3 of 10 died later. C3H.K must bring into the cross the factor for growth of E9514 which C3H.NB lacks. Presumably this is *H-2*^{k}. However, as we have pointed out above, the same results can theoretically be produced by an allele other than *H-2*^{k} but sharing some of its antigenic components. The test cannot of itself provide conclusive evidence for a new locus, but a negative result is final proof that the members of the coisogenic pair under test differ at *H-2* and not at some new locus so that every line presumed to be *non-H-2* must pass it.

TABLE 7.—*Tests with the coisogenic trio C3H, C3H.NB, and C3H.K for* H-2^k *and* H-1^a

F_1 hybrid or pure strain host	Test for:	Fraction dying*		Conclusions
		Deaths at 21 days	Final deaths	
C3H.NB $\times$ C3H.K...	*H-2*^{k} and *H-1*^{a}	17/17	17/17	C3H.K probably *H-2*^{k} C3H.NB probably *H-1*^{a}
C3H.K $\times$ DBA/1.....	*H-1*^{a}	10/10	10/10	DBA/1 probably *H-1*^{a}
C3H.K $\times$ C57L.....	*H-1*^{a}	0/17	10/17	C57L *not H-1*^{a}
C3H.....	Control	10/10	10/10	
C3H.K.....	Control	0/10	3/10	

*All mice received C3H embryo, 4 million cells per mouse intraperitoneally, and then C3H tumor E9514, 125,000 cells per mouse, 14 days later. Age at tumor inoculation = 12 to 17 weeks.

Table 7 also shows the results of applying the F_1 test, with C3H and C3H.K as the test pair, to the typing of strains DBA/1 and C57L for *H-1*^{a}. The former is indicated as having *H-1*^{a}, the latter as lacking it. We shall present a great deal more data of the same sort in a future publi-

cation on *H-1* (several years may be needed to complete some of the tests). We merely mention here that repeated tests have shown that *H-1*^{a} is shared by the 3 strains C3H, DBA/1, and DBA/2 but is lacking in all other strains tested, among them BALB/c. Since DBA/2 and BALB/c are both *H-2*^{d}, the fact that a test with the strain pair C3H and C3H.K reveals a histocompatibility *difference* between them is evidence that the difference between the test pair is not at *H-2*.

By all odds the most conclusive way to show that two histocompatibility genes are different is to prove that they occupy different positions in the linkage map. Tests to refine the measure of linkage between *H-1* and albinism (*c*) were therefore undertaken. Two crosses were used, (C3H × C3H.K) × C3H.K and (DBA/1 × C3H.K) × C3H.K. (It will be remembered that C3H.K is an albino line.) The offspring were immunized with C3H thymus and inoculated with C3H tumor E9514, 125,000 cells per mouse. Both crosses are of the backcross type. The second one uses the fact that DBA/1, like C3H, is *H-1*^{a}, and has the advantage that the F_1 and backcross mice show heterosis, although the C3H.K parent in the final cross is coisogenic with the tumor donor and hence limits effective segregation for resistance to 1 gene.

The results are shown in table 8. They are tabulated in two ways: 1) on the basis of animals dead at 35 days and 2) on the basis of final deaths. C3H controls receiving either 125,000 or 25,000 cells were all dead at 35 days. Twenty C3H.K controls all survived. At 35 days, out of a total of 137 backcross mice, 5 albinos and 53 colored mice had died; 5 colored and 74 albinos had survived. Separating these according to the sex of the heterozygous parent, we find 7.1 ± 4.5 percent recombinations among 126 offspring of heterozygous females, 9.1 percent in 11 offspring from heterozygous males. At the termination of the experiment, 17 additional albino mice and 2 additional colored mice had died. Since we do get occasional deaths among immunized C3H.K's, most or all of these late

TABLE 8.—*Demonstration of linkage of histocompatibility-1* (H-1) *and albinism* (c)*

Heterozygous parent	Number of mice dead (+) at	Offspring from backcross *H-1*^{a}*C*/*H-1*^{b}*c* × *H-1*^{b}*c*/*H-1*^{b}*c*†				Controls: fraction dying		
		Tumor dose: 1,000 × 5^3				5^3 C3H.K	3^3 C3H	5^3 C3H
		C+	*C*−	*c*+	*c*−			
♀	35 days	51	5	4	66	0/10	10/10	10/10
	84 days‡	53	3	20	50	0/10	10/10	10/10
♂	35 days	2	0	1	8	0/10	10/10	10/10
	49 days	2	0	2	7	0/10	10/10	10/10

*Recombination percent, as indicated by data at 35 days, 7.1 ± 4.5 percent in females, 9.1 percent in males (11 mice).

†One hundred and five of the 126 backcross mice whose heterozygous parent was a female were derived from the cross (DBA/1 × C3H.K) × C3H.K. All other backcross mice came from the cross (C3H × C3H.K) × C3H.K. All backcross and control mice were immunized with C3H thymus, 10 million cells per mouse, and inoculated 10 or 11 days later with C3H tumor E9514. Mice mostly 11 to 14 weeks old at tumor inoculation, a few 16 to 18 weeks.

‡Except for 8 mice killed at 49 days.

deaths probably represent false positives. The figures at 35 days probably give a rather good estimate of the crossover percent. In any event, the existence of a linkage between a histocompatibility gene and albinism is unquestionable.

We have evidence from another source to be reported at some future date that *H-1* is between albinism and pink-eye, or, as a less likely alternative, that there are two histocompatibility genes in this region of the first linkage group.

Histocompatibility-3

Histocompatibility-3, like histocompatibility-1, was first recognized in, and is defined by, a coisogenic strain pair with a visible difference (*31*). C57BL/10 is $aH\text{-}3^a/aH\text{-}3^a$ (a = nonagouti or black coat, linkage group V); B10.LP is $AH\text{-}3^b/AH\text{-}3^b$. This strain pair has now been put to additional tests similar to those used with C3H and C3H.K. The results are given in tables 9, 10, and 11. These together with their footnotes are largely self-explanatory.

The F_1 test (table 3, case 4; table 9) indicates, but cannot conclusively prove, that B10.LP, like its coisogenic mate C57BL/10, is $H\text{-}2^b$. The difference is probably not at *H-2*.

TABLE 9.—*Tests with the coisogenic trio C57BL/10, B10.D2, and B10.LP for* H-2^b *and* H-3^a

F_1 hybrid or pure strain host	Test for	Fraction that died*	Conclusions
B10.D2 × B10.LP.....	$H\text{-}2^b$ and $H\text{-}3^a$	10/10	B10.LP probably $H\text{-}2^b$ B10.D2 probably $H\text{-}3^a$
B10.LP × LP.RIII.....	$H\text{-}3^a$	0/16	LP.RIII *not* $H\text{-}3^a$
C57BL/10.....	Control	10/10	
B10.LP.....	Control	2/10	

*All mice received C57BL/10 embryo by trocar on the left side, and then C57BL/6 tumor C1498 on the right side, 125,000 cells per mouse, 14 days later. Age at tumor inoculation: 10 to 13 weeks.

TABLE 10.—*Demonstration of linkage of histocompatibility-3* (H-3) *and agouti* (A)*

Offspring from backcross ♀ $H\text{-}3^a a/H\text{-}3^b A$ × ♂ $H\text{-}3^b a/H\text{-}3^b a$†				Controls: fraction that died				
Tumor dose: 1,000 × 5^4				5^4	5^4	5^4	5^3	5^2
$A+$	$A-$	$a+$	$a-$	B10.LP	B10.LP-a	C57BL/10	C57BL/10	C57BL/10
4‡	22	31	4	2/10	0/14	10/10	19/19	18/20

*Recombination percent in heterozygous females, 11.5 ± 6.4, or, omitting the one long survivor in the $A+$ recombination class, and the one $a-$ mouse proved genetically susceptible (table 11), 9.8 ± 6.4.

†Forty of the backcross mice came from the cross (C57BL/10 × LP) × B10.LP-a, 21 from the cross (C57BL/10 × B10.LP) × B10.LP-a. All backcross and control mice were immunized with C57BL/10 thymus, 10 million cells per mouse, and inoculated 11 days later with C57BL/10 tumor S913. Age of backcross mice at tumor inoculation, 12 to 15 weeks, of controls, 10 to 18 weeks. Mice that died of tumor are recorded as +, survivors as −.

‡One of the 4 mice in this class survived 9 weeks—2 weeks longer than any other backcross mouse that succumbed to the tumor. This may indicate that it was genetically resistant.

TABLE 11.—*Evidence that backcross ♂395 from cross shown in table 10 was not a crossover*

Mouse number	Phenotype	Mated to:	Offspring*		Indicated genotype
			+	−	
♂383	*A*−	B10.LP (*H-3^{b}H-3^b*)	0	20	*AH-3^b/aH-3^b*
♂398	*A*−	"	1	19	*AH-3^b/aH-3^b*
♂395	*a*−	"	12	16	*aH-3^a/aH-3^b*

*All mice were immunized and received C57BL/10 tumor S913 (*H-3^{a}H-3^a*); + = succumbed to progressive tumor growth; − = survivors.

During the production of line B10.LP, there was a close and obvious association between agouti (*A*) and resistance. However, recombination occurred at least once, and a resistant, nonagouti line, B10.LP-*a*, was established. This made it possible to set up the linkage test in the form of a backcross (table 10). There were 8 recombinants in 61 mice. However one *A*+ (+ = died from tumor) mouse lived longer than any *a*+ mouse and was probably a false positive; one *a*− mouse was shown by a genetic test (table 11) to be genetically susceptible. There were thus not more than 6 crossovers. The indicated crossover percent is 9.8 ± 6.4, and this should be taken as a maximum figure.

The Coisogenic Strain Pair, AKR and AKR.ALB

As already noted, both members of this strain pair appear to be *H-2^k* (table 3, case 19) indicating that AKR.ALB is not an *H-2* line. Additional tests tend to confirm this and to show that it is also neither *H-1* nor *H-3*. Further studies are in progress and, when these are completed, we expect to make this strain pair the subject of a separate paper.

The Native Strain is a Slightly More Favorable Host for Tumor Transplants Than the F_1 Hybrid

It is an accepted dictum or law of transplantation that the F_1 hybrid between two inbred strains is susceptible to transplants from either parent (*7*). There is no doubt that this is consistently true of transplantable tumors under the usual experimental conditions and, though the number of tests with skin has been far fewer, of skin grafts also (*32–35*); yet in several tests in the present and in an earlier (*3*) series, with tumors given to F_1 mice at limited cell doses, we have consistently found a little less, or slower, growth than in the susceptible parent, and in one case the degree of difference has been quite striking. C57BL/6 tumor C1498, tested in C57BL/10 and (C57BL/10 × 129)F_1 at doses of 1,000, 25,000, and 625,000 cells, killed more rapidly, on the average, in the pure strain. The difference was greatest at the lowest dose; all but one C57BL/10 receiving 1,000 cells was dead by 22 days, whereas 12 of 20 F_1's were still alive. Strain A tumor SaI-A given to A's, and to F_1's from the cross A × C57BL/10, at doses of 40 and 200 cells, killed 76 percent of 58 A's and only 42 percent of 65 F_1's (combined results of intra-abdominal and

subcutaneous inoculations). At a dose of 1,000 cells per mouse, all mice of both genotypes died, but the average survival time was less in the A's.

By all odds the most striking difference in susceptibility between the native strain and the F_1 hybrid was noted with C57BL/10 tumor S913 tested in C57BL/10 and (C57BL/10 × C3H)F_1. Data from the first experiment which showed this are given in table 12. At 25,000 and 125,000 cells per mouse, there were 8 out of 10 and 10 out of 10 deaths, respectively, in the native strain, 0 out of 10 and 0 out of 5 in the F_1. When the 15 F_1's were reinoculated at 625,000 cells per mouse, 5 succumbed. Leukemia C1498 gave the same effect but to a considerably lesser degree (table 12).

TABLE 12.—*Comparison of susceptibility of parental strain and F_1 hybrid to C57BL tumors C1498 and S913*

Host*	Tumor	Fraction mice that died at different tumor doses		
		25,000	125,000	625,000
C57BL/10	C1498	10/10	10/10	10/10
C57BL/10 × C3H	C1498	11/16	10/10	
C57BL/10	S913	8/10	10/10	10/10
C57BL/10 × C3H	S913	0/10	0/5	
Same mice reinoculated	S913			5/15

*All mice pretreated with C57BL/10 thymus, 10,000,000 cells per mouse.

This experiment was repeated with larger numbers, including 68 B10.LP's and several added variables. The results (data not given in table) warrant the following conclusions: 1) There is no clear difference between the reciprocal F_1's ♀C57BL/10 × ♂C3H and ♀C3H × ♂C57BL/10; both show far less growth of S913 than the native strain. 2) The F_1's are much less favorable hosts than unimmunized B10.LP's, although the former are presumably genetically compatible and the latter are known to be incompatible due to an *H-3* difference. 3) There is a suggestion that this difference is greater at 25,000 cells than at 625,000 cells—the deaths of F_1's increased with dose and the deaths of B10.LP's decreased—but the numbers are not sufficient to prove that this is a real difference. 4) The B10.LP's respond to immunization with C57BL/10 thymus by greatly decreased growth, but this treatment did not modify the response of the F_1's. The incompatibility of the F_1's seems not to have an immune basis. The growth of S913 in 5 of 15 reinoculated F_1's in the first experiment also lends support to this conclusion.

When E9514, a transplantable leukemia from the C3H parent, was tested in the same F_1 hybrids and in pure C3H, 125,000 cells per mouse, there was the usual slight prolongation of survival of the hybrids, but no mice survived.

As an explanation of the pronounced disadvantage of the hybrid as a host for S913, we are inclined to turn to a percipient suggestion of Hauschka and Furth (*36*): "Not all the genetic determinants of graft survival

need be cellular antigens of the blood group variety. Although the *H-2* complex and other histocompatibility loci function through immunological mechanisms, metabolic traits or hormonal levels under genetic control, by which the inbred donor strain differs from the recipient, may also manifest themselves as graft dependence." The nature of the genetic factor in the present instance could only be determined by tests in segregating generations and for such tests we have no present plans. That the effect is manifest in survival of the hybrid sets it apart from the action of histocompatibility genes and should make inapplicable in this instance the further suggestion of Hauschka and Furth that "Such humoral or nutritional needs of the graft could, on occasion, mimic the segregation of isoantigens in the test hybrid." It is also clearly separable from the action of histocompatibility genes in that the incompatibility is not increased by immunization.

The significance of an F_1 effect for the present study is that it must be borne in mind in interpreting irregularities in the F_1 typing test.

How Nearly Coisogenic With Their Inbred Mates Are the Resistant Lines?

When the production of IR lines was being planned, we calculated the rate at which coisogenicity is approached in the relatively simple situation where two histocompatibility genes are segregating in the initial cross, and Professor Haldane, in an appendix, contributed a very beautiful solution of the problem for larger numbers of genes (*1*). While it is impossible to pick any particular degree of approximation to the coisogenic state and say that this is the necessary minimum, it seemed that 12 G generations (6 matings to the pure line) were the least that were likely to be acceptable. Since the necessity, in alternate generations, of mating mice that had survived a tumor graft, sometimes with regression of a large tumor mass, placed a considerable strain on the vitality of some of the stocks and made continued breeding difficult, most but not all of the lines were in fact stopped at this point. How satisfactory are these lines for the purposes to which they have been and will be put?

There was one unintended and unforseen factor that helped to hasten the approach of the coisogenic state, namely, the dominant role of the *H-2* locus in susceptibility and resistance, particularly when the test system is an old and established transplantable tumor. Survival in many of the crosses was determined largely by one locus. Since, as Professor Haldane's calculations showed, the production of the coisogenic state speeds considerably as the number of active histocompatibility genes is reduced, this situation was favorable to its rapid attainment.

This is theoretical evidence. What observational evidence is there in regard to the coisogenicity of the lines? In 4 of the 26 lines of table 3, a color gene not linked (so far as we know) with any histocompatibility gene was still segregating at G 12 or later. Line C3H.NB threw non-agouti (*aa*) mice in G generations 12 and 16, and lines AKR.K, B10.D2 and RIII.P all produced brown (*bb*) mice in G 12 or later. This is not a

bad showing considering the number of lines and the number of gene differences in the original crosses, but may indicate some selective advantage for heterozygotes. Line RIII.P has been dropped and the other 3 lines rendered coisogenic at these color loci by genetic testing and/or additional G matings.

We have already mentioned the failure of line RIII.P to give a consistently susceptible F_1 in crosses to line LP.RIII (table 4, case 9), and have interpreted this as evidence that RIII and RIII.P are not coisogenic. Strains C57BL/10 and B10.BY are another pair that has reacted in F_1 tests as if not coisogenic. We have already noted that B10.BY responded in the F_1 test (table 3, case 3) as if *H-2*^{b}, and hence presumably not an *H-2* line since C57BL/10 is *H-2*^{b} also. This has been confirmed in the more sensitive F_1 test with prior immunization. However, in a parallel F_1 test with strain B10.LP, some animals reacted as if *not-H-3*^{a}, others gave negatives in a ratio that suggested they were heterozygous for *H-3*^{a}. This line has been put through additional G generations and is now being inbred. There is a possibility that it will ultimately serve to define a new locus.

The remaining 24 IR lines in table 3, with the possible exception of AKR.K (case 1), reacted in the F_1 test as if coisogenic. It should be emphasized here that the occurrence of survivors in a "susceptible" F_1 is evidence of some departure from the coisogenic state (barring a situation like the unusual reaction of an F_1 to S913 described), but the occurrence of some deaths in a "resistant" F_1 is not. It is possible, however, that the relatively large number of cells given in these tests, which were done by the trocar method, served to obscure weaker degrees of resistance. Table 13 gives the results of a test in which various "susceptible" and "resistant" F_1 combinations involving strain C57BL/10 and two of its coisogenic sublines, with and without prior immunization, were given cell suspensions of C57BL tumor C1498 at various dose levels from 40 to 3,125,000 cells per mouse. The combinations were: case 1, the susceptible F_1, C57BL/10 $\times$ 129; case 2, the F_1, B10.D2 $\times$ B10.LP, genotype *H-2*^{d}*H-2*^{b} *H-3*^{a}*H-3*^{b}, presumed to be compatible with the tumor which is *H-2*^{b}*H-2*^{b} *H-3*^{a}*H-3*^{a}; case 3, the same with prior immunization; case 4, the F_1, B10.D2 $\times$ 129, genotype *H-2*^{d}*H-2*^{b}, compatible with the tumor unless the *H-2*^{b} allele in strain 129 is a minor variant of the *H-2*^{b} allele in C57BL/10, in which case there would be partial incompatibility due to this cause; case 5, the same with prior immunization; case 6, the resistant F_1, B10.LP $\times$ LP, genotype *H-3*^{b}*H-3*^{b}; case 7, the same with prior immunization.

Case 1, with all mice dying at 1,000 or more cells per mouse, represents the most susceptible combination. Case 2 was slightly but definitely more resistant; doses of 25,000 or more cells killed all mice but there were survivors at all lower doses. Case 4 and 6 provide an interesting comparison. At doses of 625,000 and 3,125,000 cells all mice of both genotypes succumbed; at lower doses the "resistant" genotype of case 6 gave consistently more deaths than the "susceptible" genotype,

TABLE 13.—*Percent deaths from C57BL/6 tumor C1498 given at different dose levels to "susceptible" and "resistant" F_1 hybrids, with and without prior immunization*

Case No.	F_1 host*	Immunization†	Tumor dose 1000 ×							
			5^{-2}	5^{-1}	5^0	5^1	5^2	5^3	5^4	5^5
1	C57BL/10 × 129.....	Yes	0(8)	—	100(10)	—	100(10)	—	100(10)	
2	B10.D2 × B10.LP.....	No	0(8)	0(10)	60(10)	71(7)	100(9)	100(10)	100(7)	100(6)
3	B10.D2 × B10.LP.....	Yes	0(9)	10(10)	25(8)	80(10)	100(8)	100(10)	100(9)	100(9)
4	B10.D2 × 129.....	No	—	—	40(20)	20(20)	55(20)	52(21)	100(21)	100(21)
5	B10.D2 × 129.....	Yes	—	—	11(9)	33(9)	56(9)	30(10)	100(11)	100(9)
6	B10.LP × LP.....	No	—	—	90(10)	100(10)	80(10)	80(10)	100(10)	100(10)
7	B10.LP × LP.....	Yes	—	—	40(10)	14(7)	55(11)	78(9)	80(10)	40(10)

*The hosts for case 1, 2, and 3 are "susceptible"; the hosts for case 4 and 5 are "susceptible" if C57BL/10 and 129 are both *H-2*^{b}; the hosts for case 6 and 7 are "resistant." See text for further details. Age of hosts 7 to 10 weeks at tumor inoculation.

†Immunized mice received C57BL/10 thymus, 1 million cells per mouse intra-abdominally, 9 days prior to tumor inoculation.

case 4. This was in unimmunized mice. With immunization, the potential resistance of the *H-3*^{b}*H-3*^{b} genotype (case 7) was revealed—there were survivors at all dose levels—whereas the response of the susceptible genotypes (case 3 and 5) was unaltered. It should be noted that the immunization was somewhat less than optimal since the dose was only 1 million cells per mouse and the age of the mice 7 to 10 weeks at tumor inoculation.

Other experiments similar to the one on which table 13 is based, although some of them included only immunized mice, have been carried out with the coisogenic strain pairs or trios A and A.SW; AKR, AKR.K, and AKR.ALB; and B10.D2 and C57BL/10 with B10.D2 as the tumor donor. The results have been generally the same. In nearly all instances the pure strain to which the tumor was native, or the susceptible F_1 with the tumor donor as one parent, has given 100 percent deaths at lower doses than the susceptible hybrid produced by crossing two complementary resistant strains. However, all mice of the latter hybrid type have always succumbed when the dose was carried to a level somewhere between 25,000 and 625,000 cells. Immunization with B10.D2 embryo given by trocar did raise the resistance of the "susceptible" hybrid (C57BL/10 $\times$ BALB/c) so that 625,000 instead of 25,000 tumor cells per mouse were required for complete lethality, but this is the only instance in which immunization has had any effect on "susceptible" hybrids.

Returning now to the data in table 3, which we may take as typical of all these tests, we cannot be sure of the reason for the difference between case 1 and 2, but some residual genetic contamination in the isogenic resistant strains B10.D2 and B10.LP would seem to be the most likely explanation. That the (B10.D2 $\times$ B10.LP)F_1 was a little more susceptible than the (B10.D2 $\times$ 129)F_1 can be explained by the fact that isogenic chromosome areas in B10.D2 should "cover up" contaminant areas in B10.LP, and *vice versa*. But if the slight resistance of the susceptible F_1's was due to genetic disparity, with the same net effect in unimmunized mice as the *H-3* difference of the resistant F_1's, why did they not respond to immunization in the same way that the resistant F_1's did? Without immunization the difference was slight (case 2 as compared with 6) or nonexistent (case 4 and 6); with immunization it became very clear. Perhaps the explanation is that in case 2 and case 4 there were multiple very "weak" differences, and in case 6, a single stronger difference. The immunization dose of individual antigens may have been below the threshold level in the former instances even though the tumor with its capacity for growth must have evoked some immunity, otherwise there would have been no more survivors at the lower doses than for the susceptible F_1 with C57BL/10 as one parent.

If this explanation is correct, it has interesting implications for the number of histocompatibility loci. The upper figure that genetic tests have indicated is usually placed at about 14 (*7*, *35*, *37*). If this is a valid figure, it hardly seems likely that multiple, very weak, differences would

persist in the 3 coisogenic pairs appropriately tested in addition to the one strong difference that must have been largely responsible for the occurrence of survivors during the production of the lines. Does this mean that some of the rejection of grafts in the segregating generations used in genetic tests is really due to the combined action of a number of very weak histocompatibility genes? Wright (*38*), in analyzing some of the early genetic studies with tumor transplants, has pointed out that the figures for histocompatibility gene number arrived at in these studies are probably an underestimate, and Prehn and Main (*37*), on the basis of delayed reactions in some of their skin grafts, have concluded that there are probably numerous, very weak, loci.

The implication of our results for the "purity" of the coisogenic lines is clear enough; there may be some minor contamination in most of the lines but it is not sufficient to invalidate their use in genetic tests. Their suitability for other types of tests will have to be judged in relation to each separate test situation. We are putting several of the lines through additional G generations to increase their approximation to the coisogenic state.

Additional evidence of the purity of the coisogenic lines comes from a test of several *H-2*^{d} lines, coisogenic, or presumed to be coisogenic, with C57BL/10 (table 14). Given B10.D2 (*H-2*^{d}) tumor S842, with and without prior immunization, all lines tested accepted the tumor as readily as the native strain except B10-*x* (*39*, *40*). Mitchison has already shown that B10.D2 and B10-*x* (also called C57BL/10-*H-2*^{d}) react differently (*41*), though genetic and serologic tests agree in indicating both to be *H-2*^{d}. Either the *H-2* allele in B10-*x* has unsuspected differences from that in B10.D2 or B10-*x* carries one or more differences at other loci. Pending further clarification, it would seem advisable to call this strain by the noncommittal designation, B10B, which was first used by Dr. Borges but was later dropped in favor of the more specific designation B10-*x* or C57BL/10-*H-2*^{d}.

Another and excellent test of the coisogenic state is the ratio of susceptible to resistant animals in an appropriate segregating generation,

TABLE 14.—*Test of isogenicity of 5* H-2^d *sublines of C57BL by means of transplants of B10.D2 tumor S842**

Strain	Fraction that died	
	Unimmunized	Immunized†
C57BL/10Sn (control)	1/5	
B10.D2	5/5	9/9
B10.D2-*b*		4/4
B10.SW	5/5	8/8
C57BL/6Ks-*H-2*^{d}	6/6	7/7
B10-*x*	8/8	1/6

*All mice received S842 by trocar on right side except the B10.D2-*b*, which were given S842 by cell suspension, 125,000 cells per mouse.

†Mice immunized with B10.D2 embryo, subcutaneously by trocar on the left side, 22 days prior to tumor implantation, except B10.D2-*b*, which received B10.D2 thymus, 1 million cells per mouse intra-abdominally, 15 days prior to tumor implantation.

especially with prior immunization. Strain pairs AKR and AKR.ALB (unpublished data), C3H and C3H.K (table 8), and C57BL/10 and B10.LP (table 10) were subjected to this test incidental to linkage studies and made an excellent showing. The one proven "false negative," ♂395, in the last instance, and the deficit of positive offspring from this male (table 11), may mean a low order of contamination in strain B10.LP or may be due to a slight incompatibility between tumor S913 and its native strain (*3*).

A test of homozygosity but not of coisogenicity is provided by the growth of transplantable tumors during the first few transplant generations. Without giving the data, we may state that tumors induced, or arising spontaneously, in IR lines grew essentially as well as similar tumors arising in inbred lines. There were some negatives during the first 5 transplant generations, particularly with methylcholanthrene-induced tumors (*10*), but not noticeably more in the IR lines than in the inbred.

Summary of Information on the H-2 *Locus*

Table 15 presents a condensed summary of the current state of our knowledge concerning the *H-2* locus. The information is drawn from the studies of Allen (*22*), Amos and coworkers (*25*, *26*, *28*, *42*), Gorer and coworkers (*24*, *27*), Hoecker and coworkers (*16*, *23*, *29*, *30*), and Snell and coworkers (*5*, *14*, *43*, and this paper). The following explanatory comments are in the nature of footnotes to the table. These are grouped to correspond to the 3 vertical columns of the table.

The allele *H-2*^{e}, recently reported by Hoecker *et al.* (*30*), has not been tested against the alleles *H-2*^{c} and *H-2*^{m} reported in this paper. Duplication is possible though unlikely. The same applies to *H-2*^{e} and the first two "crossover" alleles. Also it is possible, but not likely, that *H-2*^{m} is the same as the first "crossover" allele, and *H-2*^{c} the same as the second. The listed crossovers were found by Gorer *et al.* (*24*, *27*). Allen (*22*) has also reported one unquestionable crossover between D and K. It is thus a question of definition whether we wish to refer to *H-2* as a single, compound locus or as a group of closely linked loci. The former usage is probably the more convenient, but should a gene be discovered, somewhere between the various *H-2* components, whose effect was unrelated to histocompatibility, this would have to be modified or dropped. As pointed out in a previous section of this paper, C3H.NB may carry a new *H-2* allele, but because no test has been made against *H-2*^{p} it is not included in this table.

While we have referred to the new combinations of antigenic components that both Allen and Gorer have obtained from heterozygous parents as "crossovers," the possibility that they represent so-called "genic conversion" (*44*) cannot yet be entirely excluded.

Except for the D and K components, which were discovered by the histogenetic component test, all components listed were demonstrated by

TABLE 15.—*Summary of current information on the* H-2 *locus*

Allele	Antigenic components												Typed strains
H-2ª	C	D	E	F	—	H	—	K	M	—	—	—	A AKR.K
H-2ᵇ	—	D^b	E	F	—	—	—	—	—	—	—	—	A.BY C57BL/6 C57BL/10 B10.BY B10.LP C57L C3H.SW D1.LP LP 129
H-2ᶜ		D											D1.C
H-2ᵈ	C	D	E^d	F		H	—	—	M	—	—	—	BALB/c C57BL/6Ks B10.D2 B10.SW DBA/2 ST.T6
H-2ᵈ′	C	D′	E^d	F				—	M				YBL/Rr YBR/Wi
H-2ᵉ	C		E	F		—							STOLI
H-2ᶠ			E?		G	H	I						A.CA B6.M
H-2ᵏ and *H-2ᵏ′*	C	—	E	—		H		K	—	—	—	—	AKR AKR.ALB CBA CHI C3H C3H.K C57BR/a C57BR/cd C58 D1.ST ST
H-2ᵐ		—	E					K	M				AKR.M
H-2ᵖ	C	—	E					—		P	—	—	P
H-2ᵠ	C	—	E			—		—	M	—	Q	—	B6.Y C/St DBA/1
H-2ʳ	C?	—			—		—	K					LP.RIII RIII/Wy
H-2ˢ	C	—	E		G	—	—	—	—	—	—	S	A.SW
Crossover	C	D^b	E	F				K					From $(H\text{-}2^a/H\text{-}2^b)F_1$
Crossover	C	D	E	F				—					From $(H\text{-}2^a/H\text{-}2^b)F_1$
Crossover	—	D^b	E^d	F				—					From $(H\text{-}2^d/H\text{-}2^b)F_1$

red-cell agglutination tests as adapted to the mouse by Gorer. I have included only components that appear to me to be well established by published data; a number of additional components which have been listed in preliminary reports are omitted. A minus sign (—) means that an allele was tested for a particular component and found to lack it. (Gorer has used a small letter with the same significance.) The "private" components, I, P, Q, and S, which occur only once in the table, and possibly also G, may well be allelic with D or K or some other established component, though this will be a difficult point to establish conclusively. M was identified (*42*) with an antiserum prepared in strain AKR (*H-2^k*) against strain AKR.M (*H-2^m*). Amos reports that the serum has only one antibody. This confirms the identity of *H-2^m* as a separate allele.

The column headed "typed strains" includes only strains that, as of the present writing, are still in existence. Additional IR strains, dropped subsequent to typing, will be found in table 3. Strains with alleles *H-2^k* or *H-2$^{k'}$* are listed as one group. We have reported evidence (*5*) indicating that there is probably a slight difference between the *H-2* alleles of AKR and C57BR/a, and this is the basis for the distinction. We have no further evidence bearing on this difference and have made no attempt to classify other strains as having one or the other variant, though one of the tests listed in table 6 would tend to place C3H in the same group with AKR.

We originally assigned the symbol *H-2^k* to the allele in C57BR/a, and *H-2$^{k'}$* to the allele in AKR. Since C57BR/a is now very little used, while AKR and C3H are widely used, there would be a considerable gain in convenience in applying *H-2^k* to the allele in these latter strains. We have adopted this usage in this paper, and it has also been followed by Amos (*26*). We suggest that it be accepted as standard.

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Effect of Iron and Cobalt Administration on the Iron-Containing Proteins of Rats Bearing the Walker Carcinosarcoma 256[1]

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Summary

Increasing the iron stores in rats bearing the Walker carcinosarcoma 256 by iron injections had no significant effect upon the concentrations of hemoglobin, plasma iron, liver and kidney catalase, and cytochrome c in the heart, kidney, masseters, and triceps. The simultaneous use of cobalt and iron prevented anemia in the tumor-bearing rat. Cobalt, however, failed to increase the concentrations of plasma iron, liver and kidney catalase, and cytochrome c in the heart, triceps, and masseters.—**J. Nat. Cancer Inst. 21: 879–884, 1958.**

It is generally accepted that the anemia of cancer fails to respond to iron therapy, but the effect of iron administration on catalase and the cytochromes has not been established. Fukuoka and Nakahara (*1*) have shown that the effect of a tumor extract (toxohormone) upon liver catalase can be overcome by iron supplementation, but this observation was not confirmed by von Euler (*2*). It has also been reported that there was a parallel depression in liver ferritin iron and liver catalase of mice that received toxohormone (*3*). Information is not available about the effects of iron administration on cytochrome *c*.

In certain cases cobalt has been shown to be effective in the anemia of hypophysectomy (*4*), protein deficiency (*5*), chronic renal disease (*6*), chronic inflammation (*7*), and cancer (*8*). The administration of cobalt to rats, at a level sufficient to produce a marked polycythemia, did not alter the myoglobin concentration in the experiments of Faulkner and Blood (*9*). Liver-catalase depression occurred in animals with a wide variety of experimental tumors (*10*), and cytochrome *c* concentration was shown to be lower in several tissues of the rat bearing the Walker carcinosarcoma 256 (*11*). The correction of these conditions in the tumor-bearing animal may prove to be just as important as correction of the anemia.

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Materials and Methods

The Holtzman rats used in these experiments were females of the same age, and weighed from 180 to 200 gm. The Walker tumor was given as an intramuscular injection in both *rectus femoris* muscles, according to the method of Talalay *et al.* (*12*).

Cytochrome *c* was determined by the Rosenthal and Drabkin method (*13*). The methods for hemoglobin (*14*), storage iron (*15*), plasma iron (*16*), and catalase (*17*) were described in a previous publication (*11*).

$$\text{Catalase units} = \frac{\log_{10}\frac{x_o}{x}}{tW/50}$$

x_o = titer (0.01 N $KMnO_4$) of H_2O_2 substrate at $t = 0$
x = titer of substrate at t (minutes)
W = gm. of dry tissue in the 50 ml. reaction mixture

The dry weight of tissue employed was from 3 to 5 mg. The tissue was diluted until it contained from 3 to 5 mg. of dry tissue per ml., homogenized for 2 minutes in a tight-fitting, ground-glass homogenizer, and assayed within 1 minute. Reaction times of 10, 20, and 30 seconds were used.

The iron supplementation was given in daily intraperitoneal injections of 0.25 mg. of iron as ferric ammonium citrate in water. In the first experiment, cobalt 0.25 mg. per day was also administered intraperitoneally as cobaltous chloride. The injections were started on the day of tumor inoculation and continued until the tissues were analyzed at 19 days. In the second experiment, the iron remained the same but the cobalt was increased to 1 mg. These administrations were started 5 days before tumor inoculation and continued until the tissues were analyzed on the 16th day of tumor growth. The experiment was terminated at 16 days because of the apparently poor physical condition of the rats that received cobalt. The rats that did not receive either supplement were given sham injections of vehicle.

Results

A summary of the iron-containing proteins in the tissues of rats bearing the Walker tumor after iron or iron and cobalt injections will be found in table 1. The daily administration of iron produced no changes in the body weight or the tumor weight. The simultaneous use of iron and cobalt decreased carcass weight but had no effect on the growth of the tumor. The liver and spleen weights of the rats receiving iron or iron plus cobalt remained within the usually high range found in rats bearing 19-day-old Walker tumors, and the moisture content was similar to previously published values (*11*). The total dry weight of the kidneys was not significantly different between any of the groups of rats.

Iron supplementation increased the concentration of the storage iron in the liver up to that found in the normal rat. The storage iron in the

TABLE 1.—*Effects of iron and cobalt supplementation upon rats bearing the Walker carcinosarcoma 256*

	Normal rats	Rats bearing 19-day-old Walker tumors		
		Sham injection	Iron (*0.25 mg./day*)	Iron plus cobalt (*0.25 mg./day, each*)
Number of rats	18	21	14	11
Body weight (gm.)	228 ± 3*	257 ± 7*	266 ± 13*	231 ± 6*
Tumor weight (gm.)	—	55 ± 3	54 ± 4	54 ± 2
Liver weight (gm.)	7.8 ± 0.2	15.6 ± 0.9	15.4 ± 1.0	13.3 ± 1.1
Spleen weight (gm.)	0.6 ± 0.1	3.2 ± 0.2	3.2 ± 0.4	3.9 ± 0.4
Liver storage iron (mg. Fe/gm. dry wt.)	0.68 ± 0.03	0.42 ± 0.05	0.70 ± 0.08	—
Spleen storage iron (mg. Fe/gm. dry wt.)	3.03 ± 0.21	0.58 ± 0.09	1.71 ± 0.35	—
Hemoglobin (gm. %)	15.6 ± 0.1	9.8 ± 0.2	10.7 ± 0.4	11.6 ± 0.3
Plasma bound iron (μg. %)	256 ± 8	96 ± 3	89 ± 5	75 ± 6
Liver catalase (catalase units)	5600 ± 200	2000 ± 100	1900 ± 200	1900 ± 100
Kidney catalase (catalase units)	2900 ± 200	1300 ± 100	1200 ± 100	1300 ± 100
Cytochrome c (moles/gm. dry wt. × 10^9)				
Heart	124 ± 5	92 ± 5	89 ± 6	93 ± 6
Kidney	101 ± 3	83 ± 4	76 ± 5	83 ± 3
Masseters	41 ± 3	24 ± 3	24 ± 4	23 ± 2
Triceps	20 ± 1	9 ± 1	9 ± 2	8 ± 2

* Mean ± standard deviation of the mean.

spleen of tumor bearers was increased threefold over the level found in tumor bearers without iron supplementation so that the total iron was twice that found in the normal spleen. However, the concentration was lower due to the marked enlargement of the spleen. In spite of the increased iron stores, the tumor-bearing rats that received iron failed to show a significant increase ($P<0.05$) in hemoglobin concentration. The daily administration of 0.25 mg. of cobalt to the tumor bearer increased the hemoglobin concentration above that found in the sham-injected controls ($P<0.01$).

In preliminary experiments, cobalt given to the normal rat produced a polycythemia without changing the catalase or cytochrome *c*. In the

TABLE 2.—*Effects of 1 mg. cobalt per day upon rats bearing the Walker carcinosarcoma 256*

	Normal rats	Rats bearing 16-day-old Walker tumors	
		Sham injections	Iron (*0.25 mg./day*) Cobalt (*1 mg./day*)
Number of rats	7	12	12
Final body weight (gm.)	213 ± 5*	226 ± 4*	195 ± 5*
Initial body weight (gm.)	195 ± 4	198 ± 3	196 ± 3
Tumor weight (gm.)	—	38 ± 1	37 ± 2
Change in carcass weight (gm.)	+18 ± 3	−10 ± 2	−38 ± 3
Liver weight (gm.)	6.9 ± 0.2	13.3 ± 0.3	10.7 ± 0.4
Spleen weight (gm.)	0.5 ± 0.1	2.5 ± 0.2	2.3 ± 0.2
Hemoglobin (gm. %)	15.2 ± 0.1	11.3 ± 0.2	15.5 ± 0.1
Plasma bound iron (μg. %)	350 ± 22	86 ± 7	65 ± 10
Plasma unsaturated iron-binding capacity (μg. %)	51 ± 19	73 ± 16	59 ± 14
Liver catalase (catalase units)	5000 ± 200	2000 ± 100	1600 ± 100
Kidney catalase (catalase units)	3200 ± 200	1650 ± 50	1550 ± 100
Cytochrome *c* (moles/gm. dry wt. × 10^9)			
Heart	120 ± 2	110 ± 3	100 ± 5
Triceps	18 ± 2	13 ± 1	11 ± 1
Masseters	40 ± 3	30 ± 3	30 ± 2

*Mean ± standard deviation of the mean.

tumor bearer neither iron nor iron plus cobalt had a significant effect upon plasma iron, liver or kidney catalase, or the concentration of cytochrome *c* in the heart, kidney, masseters, and triceps.

It seemed desirable to repeat the experiment with a dose of cobalt that would maintain the hemoglobin concentration near the normal level. This dose was found to be 1 mg. per day for the time intervals involved in these experiments. The results, when daily amounts of iron and cobalt were used which would maintain the hemoglobin concentration at the value found in normal rats, are shown in table 2.

Cobalt injections did not affect the growth of the tumor but significantly ($P<0.01$) depressed the carcass weight. The lower liver weight

of the tumor-bearing rats receiving cobalt might be attributed to the general loss in carcass weight. The hemoglobin concentration in the tumor-bearing rats that received cobalt was maintained at or above the concentration found in the normal animals. The cobalt injections failed to increase the concentration of plasma iron, liver or kidney catalase, and the cytochrome *c* of any of the tissues studied. The liver-catalase concentration in the tumor-bearing rats receiving cobalt was significantly depressed ($P<0.01$) compared to the tumor-bearing rats that did not receive cobalt injections.

Discussion

Correcting the anemia of the tumor-bearing rats with cobalt failed to augment the decreased liver catalase. This confirmed the work of Begg *et al.* (*18*) who found that anemia produced only a moderate loss of liver-catalase activity and was therefore not the major factor contributing to the lowered liver catalase in the tumor-bearing rat. The further decrease of liver catalase in the tumor bearers receiving cobalt may be due to a partial starvation since these rats had an increased carcass-weight loss.

Cytochrome *c* showed no response to the increased hemoglobin concentration produced by iron and cobalt administration, which emphasized the diverse nature of alterations in iron metabolism in the tumor bearer.

The hemoglobin concentration in the tumor-bearing rats that received cobalt was increased without significantly changing the plasma iron from the low concentration normally found in rats bearing the Walker tumor. The maintenance of a hypoferremia when cobalt was given to overcome the anemia produced with turpentine inflammation was shown by Wintrobe *et al.* (*7*). Freireich and coworkers (*19*) have recently reported that dogs with sterile inflammation showed definite impairment of the rate of re-utilization of iron from senescent red cells, while the rate of utilization of transferrin-bound plasma iron was unimpaired. They suggested that this resulted in hypoferremia and that the decreased plasma iron contributed to the anemia of inflammation.

The experiments of Wintrobe *et al.* (*7*) with inflammation and our results on rats bearing the Walker tumor showed that in spite of the low concentration of transport iron it can be carried at a sufficient rate to increase erythropoiesis and correct the anemias of inflammation and cancer.

Goldwasser and coworkers (*20*) have shown that rats and rabbits injected with cobaltous chloride develop a high titer of erythropoietin. They therefore suggested that erythropoietin may be useful in many types of anemias, which respond only to cobalt therapy, since it is less toxic than cobalt. Our results with cobalt suggested, however, that while the anemia was corrected, the tumor-bearing rat still had an altered iron metabolism. The importance of the other alterations, such as cytochrome *c*, catalase, and plasma iron, are not fully understood.

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Cytogenetic, Serologic, and Transplantation Studies on a Heterozygous Tumor and Its Derived Variant Sublines [1,2]

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Summary

The MSWB sarcoma has been induced by methylcholanthrene in an (A♂ × A. SW♀)F_1 hybrid mouse (genotype *H-2*^{a} *H-2*^{s}). When tested in both parental strains, the sarcoma gave rise to 24 percent progressively growing tumors in the A.SW maternal strain. No tumors were ever obtained in the paternal A strain. When tested in A.SW mice preimmunized against tissue of strain A origin, the yield of tumors obtained after the inoculation of the original line decreased to 2 percent. On the other hand, the tumors that have developed in the A.SW mice, referred to as variants, showed a very high ability to grow in A.SW mice upon subsequent testing (92% on the average) and also in preimmunized mice (86%). Serologic analysis has demonstrated that the original line of MSWB contains the following *H*-2-determined isoantigenic components: D and K (derived from strain A), SG (derived from A.SW), and E and F (common to A and A.SW). Nine variants studied did not contain D and K in detectable amounts, while S, G, E, and F were still present. This was in agreement with the transplantation tests. One exceptional variant selected in an (A.SW × DBA/2)F_1 hybrid has probably lost K only, as indicated by transplantation tests. Experiments involving the inoculation of very small cell numbers and studies on the detailed chromosome cytology of the original tumor and 10 different variants indicated that the repeated detection of variants in A.SW mice was mostly due to independent events. With the exception of 2 variants, derived from the same original inoculum pool and showing closely identical karyotypes, all of the others were different from each other and from the original tumor with regard to their detailed chromosome cytology.—**J. Nat. Cancer Inst. 21: 885–923, 1958.**

Intercellular variability in neoplastic cell populations seems to be one of the major factors responsible for the stepwise development toward increased autonomy, often referred to as tumor progression (*1–5*). In order to study this variability in genetic terms, a system would be needed

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that permits the detection and characterization of specific gene mutations at definite loci in neoplastic cells. One system of this kind could be worked out (*6*) by making use of the allelic variation at the histocompatibility-2 (*H-2*) locus in some of Snell's isogenic resistant (IR) mouse strains (*7*). In a first experimental application some sarcomas were tested (*8*), induced by the same dose of methylcholanthrene in the same tissue of F_1 hybrid mice. The hosts were derived from crosses between 2 IR lines possessing a common genetic background but differing with regard to the *H-2* locus (*H-2*^{a}*H-2*^{a} and *H-2*^{s}*H-2*^{s}, respectively). Ordinarily, such tumors grow only in F_1 hybrids isogenic with the original host but in none of the parental strains. Growth in one of the parental strains might be due to the occurrence of a mutation or a loss at the *H-2* locus derived from the opposite parental strain. While a genetic change cannot be conclusively proved in somatic cells, permanance of the change, selective compatibility of the changed tumor with its new parental host, even if preimmunized against the tissue isoantigens of the opposite parent, and the loss of serologically detectable isoantigens derived from the opposite parent are some criteria that would strongly suggest the occurrence of a genetic change.

Among the 5 tumors described in the previous paper (*8*) 3 failed to grow in any of the parental types. One sarcoma showed "nonspecific" behavior from the beginning; it grew in both parents and also in unrelated genotypes. A fifth tumor, MSWB, was of particular interest. This sarcoma has been induced in a male (A $\times$ A.SW)F_1 hybrid. It regularly gave rise to a certain proportion of variant sublines that grew in one of the parental strains (A.SW) but never in the other (A). The variants behaved in accordance with this criteria. They were specific for the A.SW strain and grew in a high percentage of the mice even if the recipients were immunized against tissues of strain A origin or against the original unchanged line of the same tumor. Serologic analysis suggested that the variant cells have lost specific isoantigenic components determined by the *H-2*^{a} factor, derived from the A strain, and present in the original tumor line.

The regular occurrence of variants of one particular type to the exclusion of others was a puzzling phenomenon. The question arose whether we were dealing with the repeated selection of a single clone or a few variant clones already present in the original population, or with independent mutational events. Were the variants cytogenetically and serologically identical with each other or were they different? Did they represent cell types present in the original stem line of the tumor or were they thrown off from the main stem line from time to time? Did the variants show any regular chromosomal features that would distinguish them from the original type? An attempt has been made in the present work to obtain an answer to these questions by a combined cytogenetic, serologic, and transplantation study.

Materials and Methods

Mice.—Four isogenic resistant (IR) lines of mice were used, which were developed by G. D. Snell (*7*). Denoted A/Sn, A.SW, A.BY, and A.CA, all 4 lines have a strain A background but differ with regard to the *H-2* locus, their genotype being *H-2^a H-2^a*, *H-2^s H-2^s*, *H-2^b H-2^b*, and *H-2^f H-2^f*, respectively. In addition, some experiments included mice of the C3H/Kl, DBA/2Kl, and C57BL/Kl strains. All strains were maintained by continuous single-line brother × sister mating. In addition, the IR lines A.SW, A.BY, and A.CA were backcrossed to the A strain after every third generation. The offspring were challenged with a tumor of strain A origin. The survivors were serologically typed and then 1 pair was selected to continue the appropriate IR subline. This was done in order to keep the genetic background of the 4 lines as closely identical as possible.

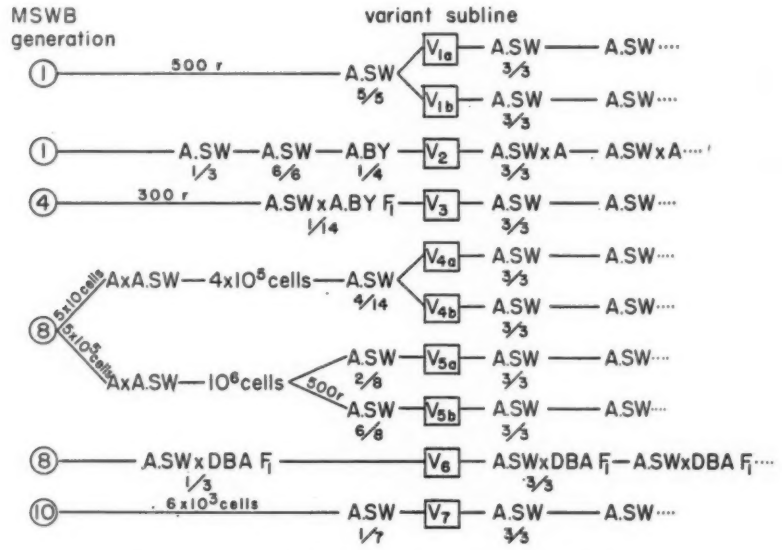
Animals of both sexes at the age of 2 to 3 months were used for the experiments. The mice were kept on a standard diet in pellet form; both drinking water and pellets were available *ad libitum*. Breeders were individually earmarked and strict pedigree records were kept. All experimental mice were earmarked at weaning, each genotype having a different mark.

Tumors.—The MSWB sarcoma was used for all experiments. It had been induced by the injection of 1 mg. methylcholanthrene into the musculature of the right thigh of a male (A × A.SW)F_1 hybrid mouse. Details on the behavior of this sarcoma after transplantation and in serologic experiments have been published previously (*8*). Two different sublines were used, which were carried separately after the second generation and have passed a total of 7 and 16 generations, respectively, in (A × A.SW)F_1 hybrids. Representative samples from early transfer generations were stored in our frozen tumor-tissue bank (*9*.) From time to time the line was cut back to an earlier transfer generation in order to avoid the progressive accumulation of serial transfers.

The tumor was tested repeatedly in both parental strains, as described previously. Since the previous paper was published, the tumor has been tested in 38 additional mice of the A strain and in 314 additional A.SW mice. None of the inoculums showed any progressive growth in A mice, while 105 A.SW mice died with large tumors. Eight of the tumors that developed in A.SW mice were studied in more detail. They will be referred to as variants and the different individual tumors tested will be denoted as V_1, V_2, etc. A schematic summary appears in text-figure 1.

V_{1a} and V_{1b}.—A cell suspension derived from the first transfer generation of MSWB was X-irradiated *in vitro* with 500 r and subsequently inoculated subcutaneously into 5 A.SW mice. All 5 animals developed tumors. Two of the 5 tumors were denoted V_{1a} and V_{1b}. They were studied further, as described under "Results." V_{1b} has been carried for 21 generations in A.SW mice.

V_2.—Another cell suspension derived from another MSWB tumor of the first transfer generation was tested in 3 A.SW mice. In one mouse a



TEXT-FIGURE 1.—History of the variant tumor lines.

tumor developed which grew on further transplantation in all of 6 A.SW mice. One of these 6 tumors was subsequently tested in 4 A.BY mice. Only 1 take was obtained. This tumor was denoted as V_2. It was carried further in (A $\times$ A.SW)F_1 hybrid mice for 15 serial transfers.

V_3.—Cells from an MSWB tumor of the fourth transfer generation were irradiated with 300 r *in vitro* and were then inoculated into 14 (A.SW $\times$ A.BY)F_1 hybrid mice. Only one of these mice developed a tumor. This variant, called V_3, was able to grow in A.SW mice (100%). The 13 (A.SW $\times$ A.BY)F_1 hybrids that remained negative after the first inoculation died with progressively growing tumors after having received a second challenge with V_3. All tumors developed at the site of the second challenge. This tumor line has been carried for 11 generations in A.SW mice.

V_{4a} and V_{4b}.—These tumors originated from the ninth transfer generation. A small inoculum of 50 cells derived from the eighth generation of MSWB was implanted into the original (A $\times$ A.SW)F_1 genotype. All 5 mice injected developed progressively growing tumors. Material was pooled from 2 of these neoplasms, and 4.5 $\times$ 10^5 cells were transferred to 14 untreated A.SW mice and another 15 animals of the same strain which were the negative survivors after a challenge with a tumor of strain A origin; they were thus preimmunized against the isoantigenic factors specifically determined by the *H-2*^{a} allele. Four of the previously untreated mice developed tumors, while no tumors developed in the immunized animals. Two of the 4 tumors were denoted V_{4a} and V_{4b} and they were carried in A.SW mice for 13 and 15 transfer generations, respectively.

V_{5a} and V_{5b}.—These variants can be traced back to the same original MSWB tumor of the eighth generation which eventually gave rise to V_{4a} and V_{4b}, except that in this case 5×10^5 cells were inoculated into 10 (A × A.SW)F_1 hybrids, all of which developed tumors. Material was subsequently pooled from 6 of the developing tumors and a cell suspension was prepared. One aliquot of the suspension received an X-ray dose of 500 r, while the remaining part was left untreated. Doses containing 1.2×10^6 cells were subsequently inoculated from both suspensions into 2 groups of 8 A.SW mice, respectively. Six out of 8 mice developed tumors from the irradiated material, while only 2 of the 8 animals were positive in the group that received the untreated cells.

Two variant tumors were studied further; 1 derived from the untreated, and 1 from the irradiated group. They were denoted V_{5a} and V_{5b} and were carried further in A.SW mice for 11 and 10 generations, respectively.

V_6.—A tumor derived from the eighth generation of the MSWB sarcoma was inoculated into 3 (A.SW × DBA/2)F_1 hybrid mice (genotype, *H-2^s H-2^d*). One of the 3 mice developed a progressively growing tumor. This was denoted as V_6. It was carried for 7 transfer generations in (A.SW × DBA/2)F_1 hybrids.

V_7.—This tumor was derived from the tenth transfer generation. A dose of 6×10^3 cells was inoculated into 7 previously untreated A.SW mice and into 13 (A.SW × A.BY)F_1 hybrids in which MSWB had previously regressed. One tumor developed in the untreated mice, while all preimmunized animals remained negative. The tumor has been carried in A.SW for 12 generations and will be referred to as V_7.

Inoculation and preservation of frozen tumors.—Solid tumors were removed aseptically and freed from macroscopically visible necrotic areas. Immediately thereafter the tissue was pressed through a 60-mesh stainless steel net into sterile Ringer's solution. In experiments in which the cell dose was not quantitated, this material was used for inoculation without any subsequent treatment. If known cell numbers had to be implanted, the crude suspension was filtered through two layers of gauze. The resulting fluid contained predominantly single cells and occasionally a few small cell clumps. The approximate viable count was estimated by Schrek's method of unstained cell counts (*10*). While stained cells can probably be considered as dead, not all unstained cells are necessarily capable of continued reproduction. The unstained cell count may therefore be considered as a maximum limit for the real viable count. It was relatively easy to bring the MSWB tumor into suspension. As a rule, about 50 percent of the suspended cells remained unstained after Schrek's eosin treatment.

The cell suspensions were inoculated subcutaneously into the right or the left flank in volumes of 0.1 ml. unless stated otherwise. The mice were examined twice a week for tumor development. Animals that remained negative or whose tumors regressed were kept under observation for a minimum of 3 months after inoculation.

A −79° C. freezing bank is routinely operated in our laboratory (*10*).

In the present case this proved very helpful, since the freezing of parallel tumor samples from the same generation facilitated repeat tests from the same original pool of material and was also useful in preventing the excessive accumulation of serial transfer generations. It also facilitated different tests at different times with the same tumor line such as transplantation to test genotypes and serologic and cytologic examinations. The frozen material was always permitted to "recover" for one passage in the genotype of origin before being used for any experiments. Freezing and thawing had no discernible effect on any of the characteristics studied. For this reason the results obtained by the use of the original unfrozen line and by repeat tests with material recovered from the tumor bank at later dates will be pooled in the present paper.

Serologic methods.—Antiserums were obtained by 4 to 5 inoculations of tumor tissue given at intervals of 10 to 15 days. The mice were bled from the retro-orbital sinus through a glass capillary 5 to 8 days after the last challenge. The amount of blood taken from each animal was 0.5 to 0.8 ml. Blood pooled from 2 or 3 mice was placed in a small pyrex centrifuge tube, allowed to clot at room temperature, and subsequently centrifuged at 800 $\times$ g for 15 minutes. The serums were collected and lyophilized in amounts of 0.05 ml. and stored in the refrigerator. The amount of tissue used for each challenge and the intervals between challenge were not strictly standardized unless mentioned otherwise. For this reason it is not possible to compare hemagglutinin titers obtained quantitatively in the different experiments.

Absorption of the antiserums was carried out by using a mixture of minced liver, kidney, and spleen, washed 3 times with 2 percent dextran solution, and packed by centrifugation. Equal volumes of tissue and antiserum were incubated at 37° C. for 45 minutes. Two absorptions were performed, as a rule. After incubation the mixture was centrifuged and the absorbed antiserum collected.

The agglutinating capacity of the antiserums was tested in the system, which contained dextran and human serum, described by Gorer and Mikulska (*11*). The dextran preparation used was Intradex Salt Free (Glaxo Ltd.), made up to 6 percent solution in 5 percent glucose. Human serum was obtained from the blood-supply center of St. Erik Hospital, Stockholm. It was collected under aseptic conditions from healthy donors. Samples of human serum were incubated at 56° C. for 1 hour. Subsequently they were absorbed, first with washed mouse tissue and the second time with washed mouse erythrocytes. The absorbed human serums were stored at −20° C.

Agglutination tests were carried out as follows: Red cells, obtained by puncture of the retro-orbital sinus, were washed twice in 0.85 percent saline and suspended to a 2 percent concentration in a 1 : 1 mixture of human serum and saline. Antiserum dilutions were made with dextran solutions diluted to 2 percent with saline. Controls contained dextran, human serum, and mouse red-cell suspensions, but no antiserum. The total volume in the agglutination tubes was 0.05 ml., consisting of 0.025

ml. diluted antiserum in dextran, or dextran alone, in the controls, and 0.025 ml. erythrocyte suspension. The mixtures were incubated at 37° C. for 1 hour. Agglutination was read exactly as described by Gorer and Mikulska (*11*).

Cytologic methods.—Tumors with large growing surfaces were produced in mice by the subcutaneous inoculation of about 0.1 ml. of cell suspension, which was distributed along 2 lines extending bilaterally from the axillary to the inguinal region. The mice were between 3 and 6 weeks of age. Approximately 10 days after transplantation, treatment was as follows: 1) an intraperitoneal injection of 0.1 ml. of a 10 percent alcohol-distilled water solution, 20 minutes; 2) an intraperitoneal injection of 0.5 ml. of a 0.025 percent colchicine solution in distilled water, 90 minutes; and 3) another intraperitoneal injection of 0.5 ml. of a 0.025 percent colchicine solution, 90 minutes. Mice were killed by cervical dislocation. The tumors were removed and finely minced with scissors, and then (*a*) bathed in 1.36 percent sodium citrate solution in distilled water for about 10 to 15 minutes at 37° C.; (*b*) fixed in chilled acetic acid-alcohol-glycerine mixture (9:3:1), 120 minutes, 4° C.; (*c*) transferred to 30 percent alcohol, 5 minutes; (*d*) transferred to distilled water, 5 minutes; (*e*) hydrolyzed in 1 N HCl at 60° C., 8 to 10 minutes; (*f*) stained in Feulgen reagent, 60 minutes; (*g*) transferred to SO_2 water, 5 minutes; (*h*) transferred to chilled 45 percent acetic acid; and (*i*) stored in 45 percent acetic acid in a deep-freeze unit at −16° C. (*12*).

Preparations were made by tapping small pieces of the stained tissue gently onto a chemically cleaned slide. A sufficient number of cells fell out to make a satisfactory preparation. It was essential to remove the lumps, as they interfered with the squashing and spreading of the chromosomes. This was usually done by pushing the drop of material to the top of the slide, then tilting and allowing the fluid to run back toward the center, leaving the coarser aggregates behind. These were removed with filter paper. The cell suspension was covered by a siliconized coverglass and squashed in the usual way. The preparations were made permanent by the dry-ice method.

Metaphases turned out to be most numerous when the colchicine treatment took place in the morning during the sleeping period of the mice, and subsequent to a previous alcohol injection. The latter proved to be very helpful in preventing the constriction of the peripheral blood vessels, which normally follows the administration of colchicine in high concentrations. It was found to be important to avoid changes in temperature during the whole procedure.

Results

Transplantation Tests

The results of all transplantation tests with the MSWB sarcoma and its different variants are shown in table 1. As in our previous experience (*8*), MSWB sarcoma grew in 100 percent of the original (A × A.SW)

TABLE 1.—*Pooled results of all transplantation*

Tumor Line	Number of passages	Host genotype				
		(A × A.SW)F_1 *H-2^{a}H-2^a*	A.SW *H-2^{a}H-2^a*	(A.SW × DBA/2)F_1 *H-2^{a}H-2^d*	(A.SW × C3H) F_1 *H-2^{a}H-2^k*	A *H-2^{a}H-2^a*
MSWB	16 in (A × A.SW)F_1	105/105 (100 %)	116/484 (24 %)	5/11	2/11	0/85
V_{1a}	2 in A.SW		9/9			0/3
V_{1b}	21 in A.SW	30/30 (100 %)	104/106 (98 %)			0/18
V_2	2 in A.SW 1 in A.BY 15 in (A × A.SW)F_1	46/46 (100 %)	72/73 (99 %)			6/74 (8 %)
V_3	14 in A.SW	15/15 (100 %)	40/41 (98 %)		4/4	0/11
V_{4a}	13 in A.SW	10/10 (100 %)	29/30 (97 %)	4/4	3/3	
4_{4b}	15 in A.SW	16/16 (100 %)	22/22 (100 %)	3/3	5/5	0/5
V_{5a}	11 in A.SW	9/10	16/16 (100 %)		3/3	
V_{5b}	10 in A.SW	4/4	20/20 (100 %)		2/2	0/3
V_6	7 in (A.SW × DBA/2)F_1		4/8	15/15	2/18 (11 %)	
V_7	12 in A.SW	4/4	24/24 (100 %)		4/4	0/13

*The figures denote the number of mice killed by progressively growing tumors as related to the total number inoculated.

†Preimmunized with C3H tissue.

‡Preimmunized with DBA/2 tissue.

§Preimmunized with A tissue or with original line of MSWB.

F_1 hybrid genotype. It consistently refused to grow in the paternal A strain, while an average of 24 percent, progressively growing tumors appeared upon testing in the maternal A.SW strain. Among the other hosts tested, the *H-2^a H-2^b* genotype (produced either by crossing the A strain with the A.BY subline or by hybridizing the A and C57BL strains) was the only one that developed progressively growing tumors in a high proportion of mice (57 and 76%, respectively). These tumors could not be carried serially in *H-2^a H-2^b* hosts, however, and were therefore regarded as false positives (*13*) rather than true variants. It would seem that the isoantigenic barrier separating the A.BY subline (*H-2^b H-2^b*) from A.SW (*H-2^a H-2^a*) is relatively weak and often insufficient to effectuate the regression of MSWB sarcoma unless strengthened by pre-

*tests with MSWB and derived variant lines**

Host genotype—Continued							
A.BY *H-2^{b}H-2^b*	A.CA *H-2^{f}H-2^f*	(A × A.BY)F$_1$ *H-2^{a}H-2^b*	(A × C57BL)F$_1$ *H-2^{a}H-2^b*	A.SW† *H-2^{a}H-2^a*	A.SW‡ *H-2^{a}H-2^a*	A.SW§ *H-2^{a}H-2^a*	A.BY§ *H-2^{b}H-2^b*
0/43	0/29	25/44 (57 %)	13/17 (76 %)	0/3	1/3	3/158 (2 %)	
			0/3				
0/9	0/13		1/3	3/3	3/3	36/39 (92 %)	
29/65 (45 %)	5/46 (11 %)	10/15	2/3	3/3	4/4	23/24 (96 %)	0/47
0/4	0/11			3/3	3/3	14/14 (100 %)	
1/5				3/3	3/3	16/36 (44 %)	
						36/43 (84 %)	
2/5				3/3	3/3	14/30 (47 %)	
1/5						28/34 (82 %)	0/4
						0/8	
				3/3	3/3	21/23 (91 %)	

vious immunization. In contrast, most tumors that developed in the A.SW maternal strain could be readily propagated in this strain. A series of direct inoculation tests performed immediately after the appearance of different individual tumors in intermediate A.SW hosts are shown in table 2. The derivation of the different variant lines is indicated. The pooled results of all inoculation tests with the serially passaged variant lines are included in table 1. While all variants could be maintained readily by serial passage in A.SW mice (V_6 was an exception, *see* later), there was a certain difference in the ability of the different lines to grow in preimmunized A.SW hosts. Some of them grew in 100 percent of the hosts or nearly 100 percent even in this case, while others only grew in a certain smaller proportion.

TABLE 2.—*First-passage tests performed with various samples of the MSWB*

	Intermediate donor type													
	A.SW													
Test genotype	1	2	3	4	5	6	7	8	9	10	11	12	13	14
(A × A.SW) F_1 *H-2^{a}H-2^s*							2/2			2/2	4/4	2/2	3/3	3/3
A.SW *H-2^{s}H-2^s*	9/9	8/9	7/8	6/9	4/9		3/3	6/6	6/6		1/5	5/5	5/5	3/3
A.BY H-*2^{b}H-2^b*														
A.CA *H-2^{f}H-2^f*							0/3				0/4		1/2†	
A *H-2^{a}H-2^a*	0/7	0/7	0/7	0/7	0/7	0/4	0/3							
(A × C57BL) F_1 *H-2^{a}H-2^b*	0/3	1/3	1/3	0/3	0/3									
(A × A.BY) F_1 *H-2^{a}H-2^b*						0/4								
Preimmunized A.SW *H-2^{s}H-2^s*							4/4			3/4				3/3
Derived variant line	V_{1a}	V_{1b}						V_2		V_7				

* Pretreated with anti-A.SW serum (*see* text).
† No growth obtained in A.CA upon further testing.

The variant line V_2 was the only one that gave rise to a certain proportion of progressively growing tumors in strains A and A.CA (table 1). These exceptional tumors, however, could not be carried serially in the corresponding strains (table 3). V_2 grew also in a fairly high proportion of A.BY hosts. Here again, serial transfer was not feasible and no takes were obtained in A.BY mice preimmunized against the original line of MSWB. Apart from the original (A × A.SW)F_1 hybrid, A.SW was thus the only host to grow V_2 regularly despite previous immunization with tissue of strain A origin. Serial transfer in (A × A.SW)F_1 hybrids for 15 generations did not result in any detectable change of the transplantation characteristics of V_2.

Among all the MSWB tumor samples that were taken from host genotypes other than the original, A.SW variants were the only ones that would breed true to type and maintain their selective compatibility in serial transfers.

sarcoma taken from intermediate hosts other than the original (A × A.SW)F₁ hybrid type

Intermediate donor types—Continued														
										(A.SW × A.BY)F₁		(A × A.BY)F¹		A*
15	16	17	18	19	20	21	22	23	24	25	26	27	28	29
2/2	1/2	2/2	2/2	1/1	2/2	2/2	2/2	3/3	2/2			5/5	5/5	2/2
3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/4	3/3	0/15	8/8	2/4	0/4	0/3
											0/3			
											0/3	0/3	0/5	
										0/5	0/3	0/5	0/5	0/5
												2/4	0/5	
4/5	3/3	1/4	3/3	4/4	4/4	3/5	1/5		5/5					0/2
V_{3a}				V_{5b}	V_{4a}	V_{4b}								

Among the variant lines that were carried serially, line V_6 is of special interest (table 1). It has been derived from an MSWB tumor that appeared after inoculation into (A.SW × DBA/2)F_1 hybrid mice (genotype *H-2ˢH-2ᵈ*). The *H-2*-determined isoantigenic constitution of this cross is presently described as $\frac{\text{CSEFG}}{\text{CDE}^{d}\text{FH}}$ (*14*). It differs from the (A × A.SW)F_1 hybrid (*H-2ˢH-2ˢ*), isoantigenic composition $\frac{\text{CDEFKH}}{\text{CSEFG}}$ only by the absence of the K component. The usual host of variant selection, the A.SW strain itself (CSEFG), differs by at least 3 components (D, K, and H being absent). Variant V_6 was regularly transplantable to (A.SW × DBA/2)F_1 hybrids (15/15) but grew only poorly (2/18) in (A.SW × C3H)F_1 hybrids $\left(\frac{\text{CSEFG}}{\text{CEHK}}\right)$ which lack component D but contain K. It grew in only 4 of 8 A.SW mice and in none of 8 preimmunized A.SW mice. In preimmunized (A.SW × DBA/2)F_1

TABLE 3.—*Inoculation tests with variant line V_2 after passage through various intermediate hosts*

	Intermediate donor type:																	
	(A × A.SW)F_1							(A×A.BY)F_1		(A × A.CA)F_1	A.BY						A.CA	A
Test genotype	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
A.SW *H-2ᵃH-2ᵃ*	2/2		10/10	10/10	5/5	1/1	3/3	6/6			5/5		2/2	3/3	5/5		3/3	2/2
A.BY *H-2ᵇH-2ᵇ*	0/2		0/10	3/5	2/5		2/4		0/2		2/2	3/4	1/3	3/4	0/3	9/13	2/2	0/3
A.CA *H-2ᶠH-2ᶠ*	0/3				0/5	1/5	0/3		0/2	0/4	2/5	0/5			0/5		2/5	0/4
A *H-2ᵃH-2ᵃ*	0/5	0/3			0/10	0/5	0/2	1/3	0/6	0/2	3/5	1/4	0/3	0/5	0/5		0/4	0/4
A.SW* * *H-2ᵃH-2ᵃ*	2/2		5/5	5/5		1/1						5/5	4/5				1/1	
A.BY* *H-2ᵇH-2ᵇ*			0/4	0/5		0/6						0/2	0/4			0/26		
A.CA* *H-2ᶠH-2ᶠ*						0/2												
A* *H-2ᵃH-2ᵃ*						0/3							0/3					

*Preimmunized with original line of MSWB.

hybrids it grew progressively and regularly (12/12). These results suggest that V_6 may have lost component K but not D. The transplantation behavior of all other variants would be consistent with the assumption that they have lost D, K, and H.

Altogether, 5 of 11 takes were obtained in (A.SW × DBA/2)F_1 hybrids after the inoculation of MSWB tumor. This is somewhat higher than that obtained after inoculation into A.SW mice (116/477) or into (A.SW × C3H)F_1 mice (2/11), though the figures are very small and the differences are not significant. Further experiments on this point are in progress.

The complete absence of any variants compatible with the paternal A strain was very puzzling. In order to increase the chance of the MSWB population to become established in A mice, use was made of the principle of "immunological enhancement" in one experiment (*15*, *16*). Strain A mice received 1 intraperitoneal injection of antiserum, produced in A mice by the inoculation of the original line of the MSWB sarcoma. Two hours after this treatment the mice were challenged with an inoculum of MSWB. Four out of 10 mice developed progressively growing tumors. Three died and 1 tumor was used for subsequent testing. No further takes were obtained in untreated A mice, however. This experiment is shown in table 2, column 29.

Quantitative Inoculations

In a previous paper (*8*) in which the preferential ability of the MSWB tumor to give rise to variants in the A.SW strain was first described, the question was raised whether this might not be due to the existence of just one or a few variant cell clones in the cell population of this sarcoma, which were present from an early stage of its inception. In this event, it might be expected that the inoculation of very small cell numbers would selectively eliminate the variant clone in the majority of cases. This was tested experimentally by inoculating cell doses ranging from about 10^7 to 30 to 50 cells. The results are shown in table 4. The decreased inoculum size had no discernible effect upon the proportion of variants, with the possible exception of one experiment (expt. 4). While preformed variant clone(s) may have been present in this particular experiment, it would appear that in the majority of experiments the variant cells were arising repeatedly *de novo*. The considerable variation in the yield of variants between different experiments would agree well with the situation that could be expected to result from a series of independent events. While the evidence is indirect, it seems to be quite incompatible with the assumption of a single pre-existing variant clone in the population. More direct proof for the independent origin of several of the variant lines will be given later in connection with the cytologic results.

Serologic Studies

Serologic experiments in a previous work (*8*) were performed with the MSWB sarcoma and some of its variants, involving both immunization

TABLE 4.—*Results of quantitative inoculations of the MSWB tumor (original line) into (A × A.SW)F_1 hybrid and the A.SW parental strain*

Experiment	Number of transfers previous to experiment	Cell No. inoculated	Progressive growth in:	
			(A × A.SW)F_1	A.SW
1	8	5×10^4	5/5	2/7
		5×10^3	5/5	1/7
		5×10^2	5/5	1/8
		5×10	5/5	2/7
2	9	6×10^3	3/3	1/7
3	3	4×10^5		11/14
		4×10^4	3/3	13/30
4	9	4×10^4		3/9
		4×10^3		4/9
		4×10^2	2/2	1/6
		4×10	3/3	0/6
5	12	1.6×10^5		3/40
		1.6×10^4	3/3	4/40
6	9	3×10^4		7/13
		3×10^3		6/13
		3×10^2	5/5	7/13
		3×10	3/5	4/13
7	5	10^7		2/12
		10^5		1/16
		10^4	8/8	1/16
8	9	1.2×10^6		2/8
9	9	4.5×10^5	2/2	4/14

and absorption tests and the determination of hemagglutination and of cytotoxic activity. These tests indicated that while the original line of the tumor contains isoantigenic components derived from both parental strains, only isoantigens specific for the A.SW parent can be detected in the variants. It is known that the A.SW strain differs from the A strain with regard to 3 isoantigenic components at least. Strain A mice can be characterized by the isoantigenic complex CDEFKH, while A.SW contains CSEFG (*14*). Thus, provided that the original MSWB line contains the complete isoantigenic set of both parental strains, it would be necessary for the variants to have lost at least 3 components, namely, D, K, and H. However, since crossing over has been reported to occur between D and K (*17*), it is probable that we are dealing with a pseudoallelic system and *H-2* is a compound rather than a single locus. This would mean that variant formation in line MSWB is a more extensive change than point mutation. To investigate the prerequisites of this interpretation in detail, it was decided to scrutinize the underlying evidence more closely in the present work by trying to determine the presence of the various individual isoantigenic components of the *H-2* system in line MSWB and its variants. The results of these investigations are shown in table 5.

The main findings can be summarized as follows:

1) Antiserums produced in A.SW mice (H-2^s H-2^s; *isoantigens CSEFG*) *by the injection of the original and the variant lines of the MSWB sarcoma.*— Antiserums produced in A.SW mice by the inoculation of the original MSWB line showed hemagglutinating activity against strain A cells (table 5). This activity could be due to isoantibodies directed against components D, K, and H. As shown in table 5, the activity of such serums was retained, although with a diminished titer, after absorption with tissue of C3H origin (CEKH), which removes the anti-K and anti-H components. The remaining activity, presumably anti-D, was absorbed completely by tissue of DBA/2 origin (CDE^dFH), as expected. When DBA/2 tissue was used for the first absorption, removing anti-D and anti-H, the serum was still active, indicating the presence of anti-K. This activity could be removed quantitatively by absorption with strain C3H tissue.

Subsequently, an attempt was made to determine whether the variants would elicit any hemagglutinin production in A.SW mice. Since they grow progressively in A.SW mice and might therefore absorb any hemagglutinins that would happen to be formed, irreversibly X-ray-damaged tumor tissue was used for inoculation. Tumor cell suspensions were treated with 15,000 r X-ray doses *in vitro* prior to implantation. Such irradiated cells are quite capable of functioning as an isoantigenic stimulus. When challenged with irradiated MSWB cells 4 times at 7-day intervals, the serum of A.SW mice agglutinated strain A erythrocytes in 1:512 dilutions. The test was performed 7 days after the last challenge. In contrast, identical amounts of the 3 variant lines V_{4b}, V_{5b}, and V_7, given at the same time intervals, did not induce any detectable activity against strain A erythrocytes.

Taken together, these results indicate that both D and K are present in the original MSWB tumor but cannot be demonstrated in any of the 3 variant lines. This conforms with the transplantation behavior of the different lines.

2) Antiserums produced in DBA/2 mice (H-2^d H-2^d; *isoantigens* CDE^dFH) *and* $(DBA/2 \times C57BL)F_1$ *mice* (H-2^d H-2^b; *isoantigens* $\frac{CDE^dFH}{BEF}$).— Antiserums produced in DBA/2 mice by the inoculation of the original MSWB line were capable of agglutinating both A and A.SW red cells. The active components in this system can be postulated as EK and ESG, respectively. After the absorption of the anti-E component with A.BY tissue (*H-2^b H-2^b*), activity was retained against both A and A.SW cells. If the original antiserum was absorbed with A.SW cells, it was still active against strain A cells; this activity was probably due to anti-K.

When antiserums were tested that had been produced against variant lines V_{1b} and V_{5b} in DBA/2 mice, absorption with A.SW tissue removed all activity against strain A erythrocytes, suggesting the absence of component K. After absorption with A.BY tissue, the serum was active against A cells. If the activity of these serums against A cells were due

TABLE 5.—*Serologic analysis of MSWB and its variants*

Isohemagglutinins produced after the inoculation of:									
Original line MSWB						Variant lines			
Host*	After absorption with*	Tested against erythrocytes of: A* Known effective components	A* Titer	A.SW* Known effective components	A.SW* Titer	After absorption with*	Tested against erythrocytes of: A* Titer	Tested against erythrocytes of: A.SW* Titer	Variant line used
A.SW	—	DKH	5120			—	0		V_{4b}[3]
	C3H	D[1]	640			—	0		V_{5b}[3]
	DBA/2	K[2]	320			—	0		V_7[3]
A	—			GS	512				
DBA/2	DBA/2	KE	192	GSE	768	DBA/2	128	64	V_{1b}
	A.BY	K	96	GS	192	A.SW	0	0	
	A.SW	K	48	—	0	A.BY	128	128	V_{5b}
						A.SW	0	0	
(DBA/2 × C57BL)F_1	—	K	512	GS	256	—	512	512	V_{1b}
						A.SW	0	0	
						A.BY	512	512	V_{4a}
						A.SW	0	0	
	A.SW	K	24	—	0	A	0	128	
						A.BY	32	32	V_{4a}
						A.SW	0	0	

C3H	C3H	DF	192	GSF	192	C3H A.SW	64 0	512 0	V_{1b}
	A.BY	D	48	GS	96	C3H A.BY A.SW	128 64 0	not tested not tested 0	V_{4a}
	A.SW	D	48	—	0	A.BY A.SW	128 0	512 0	V_{4b}
(C3H × C57BL)F_1	A.BY	D	256	GS	128	A.BY A.SW	64 0	64 0	V_{4a}
	A.SW	D	64	—	0	A.BY A.SW	256 0	512 0	V_{4b}
						A.BY A.SW	8 0	64 0	V_7

* *Strain*	*Genotype*	*Known isoantigens* (14)
A	*H-2^{a}H-2^a*	*CDEFKH*
A.SW	*H-2^{s}H-2^s*	*CSEFG*
DBA/2	*H-2^{d}H-2^d*	*CDE^{d}FH*
C3H	*H-2^{k}H-2^k*	*CEHK*
C57BL	*H-2^{b}H-2^b*	*BEF*
A.BY	*H-2^{b}H-2^b*	*BEF*

1. Subsequent absorption with DBA/2 tissue removed all activity.
2. Subsequent absorption with C3H tissue removed all activity.
3. Inoculated after X irradiation with 15,000 r *in vitro*.

to anti-E only, it could be expected that absorption with A.BY tissue would remove it. The deviating result can be explained if it is assumed that A and A.SW cells have more component(s) in common than the hitherto recognized factors C, E, and F.

The antiserum obtained after inoculations of MSWB tumor into (DBA/2 × C57BL)F_1 hybrids was active against both A and A.SW red cells. This can be attributed to components K and GS, respectively. After absorption with A.SW tissue, there was some activity left against A cells, indicating the actual presence of anti-K.

Serums obtained from similar F_1 hybrids after immunization with the variant sublines V_{1b}, V_{4a}, and V_{5a} showed activity against A erythrocytes. This was completely removed by absorption with A.SW tissue. When absorbing with A tissue, there was still activity left against A.SW red cells (anti-SG). When absorbing with A.BY tissue, there was again an unexpected activity against A cells, again indicating the existence of additional unknown component(s) common for A and A.SW cells.

3) Antiserums produced in C3H mice (H-2^k H-2^k; *isoantigens CEKH*) *and* (*C3H* × *C57BL*)F_1 *hybrids* (H-2^k H-2^b; *isoantigens* $\frac{CEKH}{BEF}$).—Antiserums produced in C3H mice by the inoculation of MSWB tumor reacted against both A and A.SW erythrocytes (presumed active components DF and SGF, respectively). After the absorption of the anti-F component with A.BY tissue, activity was retained against both cell types, due to anti-D and anti-SG, respectively). After absorption with A.SW tissue the activity against A cells was retained (anti-D). Activity of the serums produced by the inoculations of variant lines V_{1b}, V_{4a}, and V_{5b} against A cells could be removed completely with A.SW tissue, suggesting the absence of component D. There was some activity left, however, after absorption with A.BY tissue. D being absent, there is no known isoantigenic component which should be active here. A component present in both A and A.SW tissue but not in C3H and A.BY would give this type of result.

The antiserums produced in (C3H × C57BL)F_1 hybrids by the inoculation of the original MSWB line showed hemagglutinating activity against both A and A.SW cells (known factors D and SG, respectively). After absorption with A.SW tissue some activity was left, indicating the presence of component D.

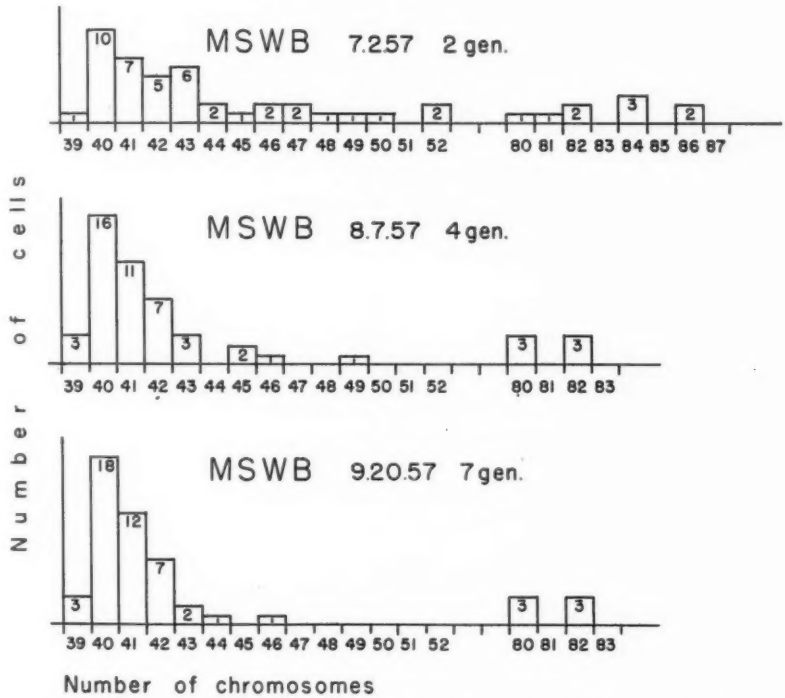
When variant lines V_{4a}, V_{5b}, and V_7 were used for producing the antiserums in (C3H × C57BL)F_1 hybrids, the activity against A cells was completely absorbed by A.SW tissue.

These results indicate that the original line of MSWB contains the isoantigenic components D and K (derived from the strain A parent), S and G (derived from A.SW), and E and F (common for A and A.SW). On the other hand, both components D and K were regularly absent from the variants, while S, G, E, and F were still present. Besides confirming the previous findings, this establishes that at least two components have been lost simultaneously from the variant cells.

Cytogenetic Data

The cytogenetic characterization of line MSWB and the variants will be given in the same sequence as their description in "Materials and Methods."

MSWB contained two stem lines, s_1 and s_2. $S_1 = 40$ chromosomes and $s_2 = 80$ chromosomes. Six samples, containing 50 metaphases each, derived from the second, fourth, and seventh transfer generation of subline I (text-fig. 2), and from the second, ninth, and eleventh passage of subline II of MSWB, respectively, have been drawn and analyzed. Out of these 300 metaphases, 265 showed the s_1 complement, while 35 metaphases contained the s_2 combination. S_1 is marked by one subtelocentric chromosome with a secondary constriction in the long arm dividing it in 2 approximately equal portions (fig. 1, A, and fig. 3). This was the third longest element in the diploid stemline s_1 (fig. 3), and it was present twice in s_2, the fifth and sixth longest chromosomes here. Four of the 265 s_1 metaphases showed a deviating complement of marker chromosomes. Two of them contained the marker twice (fig. 1, B); one was deficient for it, and another one had a long metacentric chromosome in



TEXT-FIGURE 2.—Chromosome number distribution in the second, fourth, and seventh transfer generation of MSWB, subline I. Each histogram is based on 50 metaphases.

TABLE 6.—*Distribution of MSWB stemline cells with normal and atypical marker-chromosome complement**

a) No. of chro.	39	40	41	42	43	44	45	46	47	48	49	50	51	52	80	81	82	83	84
No. of cells	2	13	10	6	3	1	2	2	2	1	2			1	3		1		1
+ 1 Ma	2	12	10	6	3	1	2	2	2	1	2			1					
+ 2 Ma		1													3		1		1
− Ma																			
+ Mc				1															
b) No. of cells	2	23	11	4	1	2									3		3		
+ 1 Ma	2	23	11	4	1	2													
+ 2 Ma															3		3		
− Ma																			
+ Mc																			
c) No. of cells	4	17	13	5	3	2	1	2		1					1		1		
+ 1 Ma	4	17	13	5	3	2	1	2		1									
+ 2 Ma															1		1		
− Ma																			
+ Mc																			

*Samples derived from a) second, b) ninth, and c) eleventh transfer generation of subline 11. No. of chro. = number of chromosomes, No. of cells = number of cells, + 1 Ma = cells with 1 marker (chromosome with secondary constriction), + 2 Ma = cells with 2 markers, — Ma = cells without marker, + Mc = cells with 1 marker and 1 additional metacentric chromosome. Based on 50 metaphases for each generation.

addition to the MSWB marker (fig. 1, C). These cells with deviating marker combinations have been found limited to only 2 tumors of the second transfer generation, belonging to the 2 different sublines (table 6). Fifty metaphases, each, of the fourth and seventh generation of subline I and of the ninth and eleventh generation of subline II, respectively (table 6), contained only MSWB stemline cells with typical marker-chromosome combinations. There was no variation of the markers in s_2.

V_{1a} and V_{1b}.—The 2 stem lines, s_1 and s_2, of V_{1a} and V_{1b} appeared to be identical with regard to chromosome number and marker-chromosome combination. The stemline $_1$ of V_{1a} and V_{1b} = 48 chromosomes; the stemline $_2$ of V_{1a} and V_{1b} = 96 chromosomes. The longest chromosome in the hyperdiploid stem line (fig. 1, D, and fig. 3) and the 2 longest elements in the hypertetraploid stem lines showed a secondary constriction in the middle of the long arm. Since the long marker chromosome in V_{1a} and V_{1b} corresponded in length and morphology to the MSWB marker, it can be suggested that they are homologous. In addition, the V_{1a} and V_{1b} stemline $_1$ contained 2 minutes (M), (fig. 1, D, and fig. 3), and V_{1a} and V_{1b} stemline $_2$, 4 M on the average.

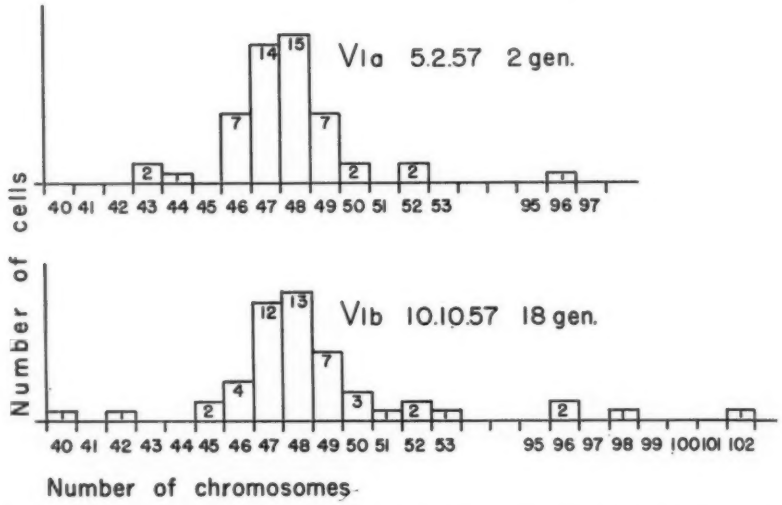
Four different transfer generations of V_{1a} (second generation) and V_{1b} (tenth, seventeenth, and eighteenth generation) were examined including 50 metaphases for each generation. Out of these 200 metaphases analyzed, 189 were hyperdiploid, 11 were hypertetraploid. Four of the hyperdiploid stemline cells were atypical with regard to the long marker chromosome. One cell was deficient for it, 1 cell had the marker twice, and 1 had it 3 times. There was no variability in this regard in stemline $_2$. The M showed greater variation. Of 189 V_{1a} and V_{1b} stemline $_1$ cells examined, 15 had one M, 145 two M, and 29, three M. Of 11 cells of V_{1a} and V_{1b} stemline $_2$ analyzed, 8 had 4 M and 3 had 5 M. The variability of the marker chromosomes was similar in the early and late transfer generations of V_{1a} and V_{1b}. The percentage of cells with chromosome numbers differing from the modal number was not found to change during serial transplantation (text-fig. 3).

V_3 was composed of 3 different stem lines: s_3 = 41 chromosomes (fig. 1, E), s_2 = 53 chromosomes (fig. 1, F), and s_1 = 87 chromosomes (fig. 1, G, and fig. 3). Out of 200 metaphases of the second, third, fifth, and eighth generation examined, 24 were stemline $_3$ cells, 38 were stemline $_2$ cells, and 138 were stemline $_1$ cells.

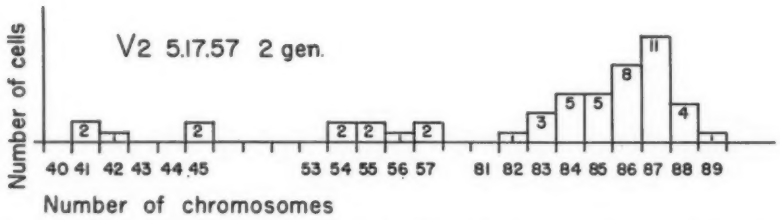
Out of the 24 hyperdiploid stemline $_3$ cells, 21 contained 2 M; 3 cells contained 3 M. Out of the 38 hypotriploid stemline $_2$ cells, 35 showed 2 M, 3 cells, 3 M. Out of the hypertetraploid stemline $_1$ cells examined, 8 cells had 2 M, 104 cells had 3 M, and 26 cells had 4 M (text-fig. 4).

V_3 was characterized by 2 stem lines, a hyperdiploid stemline s_1 with 43 chromosomes and a hypertetraploid stemline s_2 with 86 chromosomes. Out of 150 metaphases of the fourth, tenth, and eleventh transfer generation of V_3 controlled, 134 belonged to the hyperdiploid class, 16 to the hypertetraploid line.

The hyperdiploid line showed 2 markers: 1 long metacentric chro-



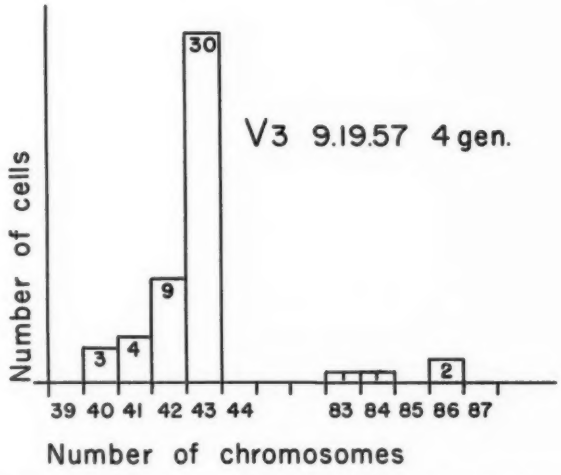
TEXT-FIGURE 3.—Chromosome number distribution in the second transfer generation of V_{1a} and the eighteenth transfer generation of V_{1b}, based on 51 metaphases for each generation.



TEXT-FIGURE 4.—Distribution of the 3 stemline cell classes in the second transfer generation of V_2; 50 metaphases examined.

mosome with arms of about equal length and 1 long subtelocentric element with a secondary constriction in the middle of the long arm, presumably homologous to the MSWB marker (fig. 1, H, and fig. 3). The metacentric chromosome was the longest and the other marker the second longest element in the set. Of the hyperdiploid class, 130 metaphases showed typical marker-chromosome equipment; 4 cells showed atypical combinations. One cell was deficient for the metacentric, 1 for the chromosome with the secondary constriction. One cell contained the metacentric marker and 1 cell contained 2 of the other marker. The hypertetraploid line showed no variation in this respect; all cells examined had 2 metacentric chromosomes with a secondary constriction (text-fig. 5).

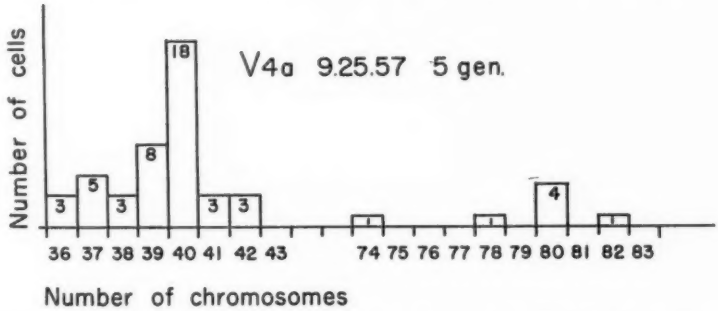
The cytology of V_{4a} was examined in 4 different transfer generations, the fifth, sixth, tenth, and eleventh. Of the 200 metaphases, 184 cells were a diploid stemline $_1$ with 40 chromosomes; 16 cells a tetraploid stem line $_2$ with 80 chromosomes. S_1 and s_2 of V_4 had no specific marker chromo-



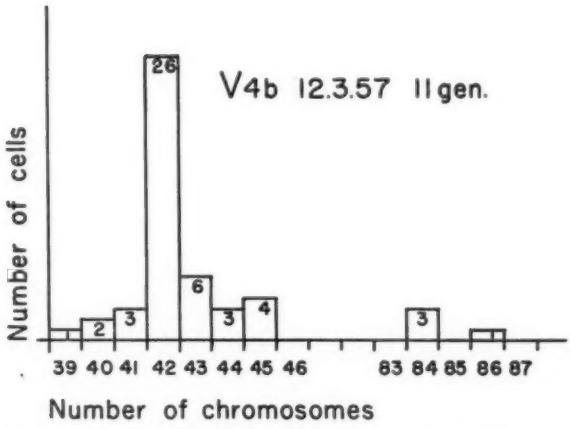
TEXT-FIGURE 5.—Distribution of the stemline $_1$ and $_2$ cells in the fourth generation of V_3. Examination of 50 metaphases.

somes (fig. 1, I, and fig. 3). One cell of the diploid class showed 1 M (text-fig. 6).

V_{4b} was a tumor with a hyperdiploid main stemline s_1 with 42 chromosomes and a hypertetraploid line s_2 with 84 chromosomes. S_1 represented 185 cells and s_2 represented 15 cells of 200 metaphases from 2 tumors, each, of the eleventh and the thirteenth generation of 2 independent transplantation lines. The hyperdiploid stem line was marked by a long subtelocentric chromosome with a secondary constriction in the long arm, presumably homologous to the MSWB line. It was the third longest element of the chromosome set (fig. 3). Furthermore, the stemline$_1$ showed 1 M (fig. 2, A, and fig. 3). The marker-chromosome combination in the hyperdiploid class was stable. Only 1 metaphase showed the long marker twice; another one was deficient for the M. In all 15 cells of the hypertetraploid line, the 2 markers were identified twice (text-fig. 7).



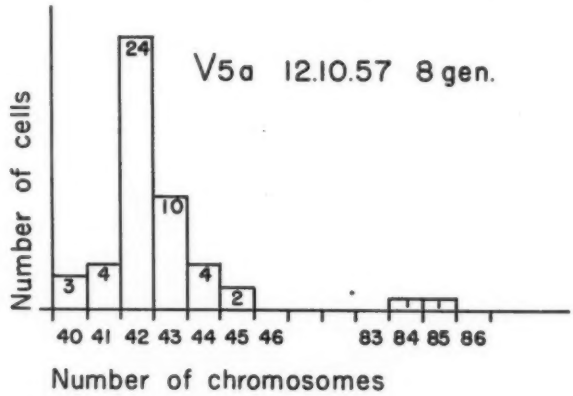
TEXT-FIGURE 6.—Distribution of the 2 stemline classes in the fifth transfer generation of V_{4a}, based on 50 metaphases.



TEXT-FIGURE 7.—Grouping of the 2 stem lines in 49 metaphases of the eleventh generation of V_{4b}.

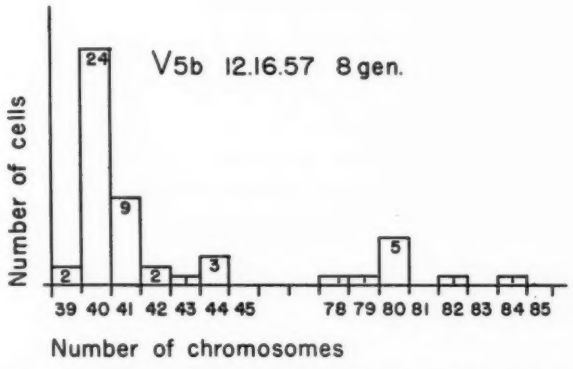
V_{5a}.—Fifty metaphases, each, of the fifth, eighth, and ninth transfer generation of V_{5a} have been investigated. Of the 150 cells, 141 examined had a hyperdiploid chromosome number showing mainly a chromosome complement of $s_1 = 42$ chromosomes. The s_1 was characterized by 1 subtelocentric long chromosome with a secondary constriction in the middle of the long arm, presumably homologous to the MSWB marker, and 1 acrocentric element that had about the same length as the MSWB marker. The 2 arms of the acrocentric chromosome showed a length proportion of about 1 : 3 (fig. 2, B, and fig. 3). All 9 hypertetraploid stemline$_2$ cells showed the double number of markers. There was no variation of the markers in s_1 and s_2 (text-fig. 8).

V_{5b} was built up by 2 stem lines: $s_1 = 40$ chromosomes, $s_2 = 80$ chromosomes. The longest element in s_1 was an acrocentric marker with an arm



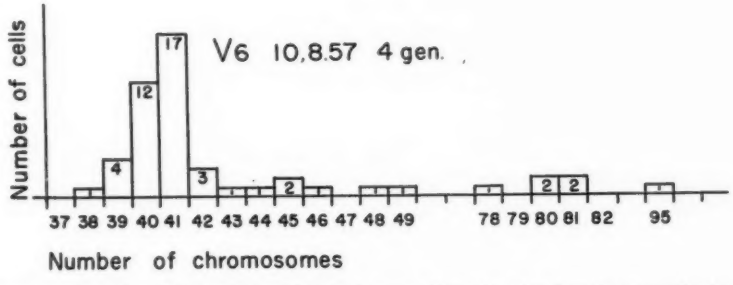
TEXT-FIGURE 8.—Distribution of the 2 stem lines in the eighth generation of V_{5a}.

length proportion of about 1 : 3 and the second longest chromosome was probably identical to the MSWB marker (fig. 2, C, and fig. 3). In s_2 both markers were identified twice in all cases. Of 150 metaphases from the sixth, seventh, and eighth generation, 123 cells belonged to the diploid s_1 group, the remaining 27 cells to the tetraploid s_2 class. S_1 and s_2 were stable with regard to their marker-chromosome combination (text-fig. 9).



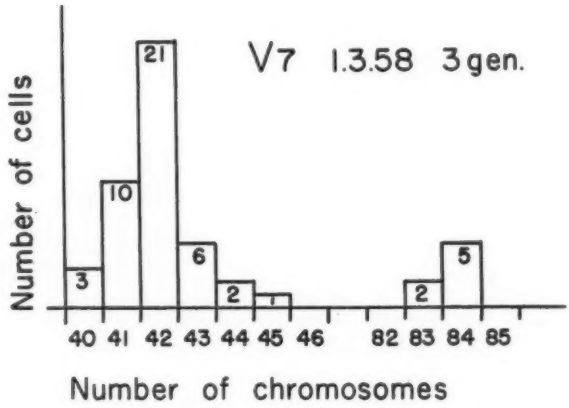
TEXT-FIGURE 9.—Distribution of the 2 stem lines in the eighth generation of V_{5b}.

V_6 showed 2 stem lines: $s_1 = 41$ chromosomes, $s_2 = 82$ chromosomes. One hundred metaphases of the third and fourth transplantation generation have been drawn. Ninety-one of them belonged to s_1, 9 cells to s_2. S_1 was marked by 3 long metacentric chromosomes of about equal length. They were about twice as long as the longest subtelocentric chromosome of the MSWB line. In addition, s_1 was characterized by a chromosome presumably identical to the MSWB marker (fig. 2, D, and fig. 3). While the chromosome with the secondary constriction was present in all cells of the s_1 class, in a single dose, the number of metacentric chromosomes varied in this class. Three s_1 cells had 1 metacentric chromosome, 4 had 2, 82 had 3, and 2 s_1 cells had 4. All 9 s_2 cells showed 6 metacentric chromosomes and 2 chromosomes with a secondary constriction (text-fig. 10).



TEXT-FIGURE 10.—Distribution of the 2 stem lines in the fourth transplantation generation of V_6; 50 metaphases examined.

V_7 was composed of 2 stem lines: $s_1 = 42$ chromosomes with 2 long metacentric components of about equal length and a long chromosome with a secondary constriction, probably identical with the MSWB marker (fig. 2, E, and fig. 3). Eighty-eight of the 100 metaphases from the third and fourth transplantation generation, which have been examined, belonged to s_1 and showed the typical marker-chromosome combination. The remaining 12 s_2 cells with the modal number of 84 chromosomes were characterized by 1 superlong subtelocentric chromosome, 3 metacentric chromosomes of about equal length, and 3 chromosomes with secondary constrictions, probably not homologous (fig. 2, F, and text-fig. 11).



TEXT-FIGURE 11.—Grouping of the 2 stemline classes in the third transplantation generation of V_7, based on 50 metaphases.

Summary of the Cytogenetic Data

The cytologic analysis of MSWB and the 10 variants has demonstrated that all variants were different from MSWB with regard to their chromosome combination. Eight of them, V_2, V_3, V_{4a}, V_{4b}, V_{5a}, V_{5b}, V_6, and V_7, showed a cytogenetic individuality, while 2, V_{1a} and V_{1b} were indistinguishable from each other, though undetectable differences in the chromosomal equipment of these 2 variants cannot be excluded. MSWB and 9 variants (V_{1a}, V_{1b}, V_3, V_{4a}, V_{4b}, V_{5a}, V_{5b}, V_6, and V_7) had a main stem line (s_1) with a diploid or hyperdiploid number of chromosomes. In addition, all of them contained a smaller fraction of stemline cells with a tetraploid or hypertetraploid chromosome number (s_2). V_2 was an exceptional variant containing 3 different stem lines: 1 hypertetraploid (s_1), 1 hypotriploid (s_2), and 1 hyperdiploid (s_3). The proportion of the stemline cells in the populations of MSWB and the different variants was characteristically different. The number of s_2 and s_3 cells seemed to be higher in earlier transfer generations than in later ones, as shown by the comparison of the second, fourth, and seventh generation of MSWB, subline I (text-fig. 2). Here the second generation contained 18 percent s_2

cells, while only 12 percent s_2 cells have been counted in the fourth and seventh generation, respectively. The second generation of subline II of MSWB included 10 percent, the ninth, 12 percent, and the eleventh generation, 4 percent s_2 cells (table 6). The percentage of s_2 cells in 300 metaphases of MSWB from 6 different transfer generations has been 11.6 percent.

The stemlines s_1, s_2, and s_3 of MSWB and the variants were characterized by distinct chromosome numbers. In the early transfer generations, however, the cells with the typical stemline chromosome number could not be distinguished with equal ease, since the number of cells showing variation of chromosome numbers around the modal number was often high and the variation broad. The MSWB population in the second transfer generation (subline I), for instance, contained only 22 percent cells with exact s_1 and s_2 chromosome numbers. The cells in the diploid class showed chromosome number variation from 39 to 52 chromosomes ($s_1 = 40$ chromosomes) and in the tetraploid class a variation from 80 to 86 chromosomes ($s_2 = 80$ chromosomes). In contrast to this, the s_1 and s_2 cells with the typical chromosome number predominated (42%) in the population of the seventh transfer generation and the variation was considerably reduced (text-fig. 2). Only the variants V_{1a} and V_{1b} maintained the same degree of variability through the second to the eighteenth transfer generation (text-fig. 3).

MSWB and its variants were further characterized by the presence of typical marker chromosomes, by combinations of different marker chromosomes, or by the lack of distinctive markers from their chromosome complement. MSWB and 8 variants (V_{1a}, V_{1b}, V_3, V_{4b}, V_{5a}, V_{5b}, V_6, and V_7) also contained 1 chromosome with a secondary constriction in the long arm dividing it in halves approximately equal in length. Since the length and the morphology of this marker was the same in line MSWB and the 8 variants, it may be that it is the same element in all of them (fig. 3). In two variants, V_2 and V_{4a}, the MSWB marker was deficient. Presumably, it has been eliminated or it has lost its morphologic identity by chromosome rearrangements during the development of the variant genotypes. Nine variants exhibited new marker chromosomes and belonged to the 3 following types: *a*) metacentric chromosomes, mostly about twice as long as the longest subtelocentric element of the MSWB set; *b*) acrocentric chromosomes of different lengths; c) minutes (M). Figures 1, 2, and 3 show the marker-chromosome equipment of MSWB and the variants. Only V_{4a} was free of detectable chromosome mutations. In general the tetraploid or hypertetraploid stem lines (s_2) of MSWB and the variants V_{1a}, V_{1b}, V_3, V_{4b}, V_{5a}, V_{5b}, and V_6 contained twice as many marker chromosomes as the corresponding stemline $_1$. Stemline $_2$ of V_7 was exceptional in this respect. It had a deviating equipment of marker chromosomes, which makes it obvious that chromosome remodeling took place during the origin or the subsequent development of this stem line. In V_2 the hypertetraploid s_1 and the hypotriploid s_2 had mainly 3 M, the hyperdiploid s_3 had chiefly 2 M.

The numerical variability of the markers in stemline$_1$ of MSWB and the variants was higher than that in stemline$_2$ and stemline$_3$ of these tumors. Three classes of tumors could be distinguished: *a*) high variability of the markers in the s_1 in the early generations, and decreased or no variation in the later ones, *b*) constantly high variability, and *c*) constantly low or no variability of the marker chromosomes in stemline$_1$ in early and late transfer generations. MSWB belonged to class *a*. Of 265 s_1 cells, only 4 showed an atypical marker-chromosome constitution. These cells have been limited to 2 tumors of the second transfer generation (86 s_1 cells). All the remaining stemline$_1$ cells of the fourth, seventh, ninth, and eleventh generation of sublines I and II were stable. V_2 can be used as an example for class *b*, with an approximately constantly high variation of the markers. Of 138 stemline$_1$ metaphases of V_2, 34 cells showed unstable marker equipment—7 of them have been found among the s_1 cells of the second generation (38 cells), 10 among the s_1 cells in the third generation (39 cells), 8 among the s_1 cells of the fifth generation (29 cells), and 9 among the s_1 cells in the eighth generation (32 cells). Class *c* tumors were represented by the variants V_{1a} and V_{1b}. Of 189 stemline$_1$ cells of V_{1a} and V_{1b} only 4 cells had atypical marker-chromosome combinations. Forty-nine s_1 cells of the second transfer generation of V_{1a} showed 1 such cell, 46 s_1 cells of V_{1b} of the tenth generation contained none of them, 48 s_1 cells of the seventeenth generation showed 2 such cells, and 46 s_1 cells of the eighteenth transfer generation of V_{1b} had 1 cell with an atypical marker-chromosome equipment.

All 9 variant karyotypes resulted from the MSWB stemline$_1$ or stemline$_2$ chromosome complement. The fact that most of the variants contained new stemline numbers with the appearance of new markers in some of them together with the absence of the MSWB marker from others showed that structural remodeling has taken place during the origin and/or the subsequent development of the variant karyotypes. It can be assumed that each observable structural change corresponds to many intra- or interchromosomal changes in many chromosomes. On the other hand, not all structural remodeling will lead to identifiable chromosome mutations. For these reasons it is impossible to determine the sum of structural variation that has taken place during the development of a variant karyotype. Cryptostructural variation might be possibly assessed from a more accurate comparison of the idiograms of MSWB and its variants (*18*), although no quantitative or qualitative inferences could be drawn.

Measurements were made on chromosome drawings from 6 metaphases of MSWB and each of the variants. The chromosomes were arranged in the order of decreasing length (fig. 3). The different position of the MSWB marker in the idiograms of MSWB and the variants V_{1a}, V_{1b}, V_3, V_{4b}, V_{5a}, V_{5b}, V_6, and V_7 made it obvious that cryptostructural variation has also been involved in the development of the variant karyotypes. Such variation may also explain the increased number of chromosomes of the shorter and longer type occurring concurrently with a diminished

number of the elements of middle length in the V_2 karyotype and the numerical reduction of the longer elements in the V_{4a} karyotype.

Discussion

It has been found previously that the MSWB sarcoma, induced by methylcholanthrene in an (A♂ × A.SW♀)F_1 hybrid mouse (genotype *H-2^{a}H-2^s*), gives rise to progressively growing tumors in about 24 percent of the maternal A.SW strain on the average, while it regresses consistently in the paternal A strain. Several of the "variant" tumors that have appeared in A.SW hosts showed a high degree of selective compatibility with this strain upon subsequent testing, *i.e.*, they grew in almost 100 percent of A.SW mice even if preimmunized against tissues of strain A origin or the original line of MSWB itself. They still refused to grow in the A strain or in other unrelated genotypes. Serologic analysis indicated that the variants no longer contained detectable amounts of the specific *H-2*-determined isoantigenic components which have been derived from the A strain and are absent from A.SW.

Since a fluctuating but still fairly high frequency of variants was obtained by different tests of the tumor, including different sublines and several transfer generations, the question arose whether we were dealing with the repeated selection of just one clone or a few clones of variant cells already present in the primary tumor or, rather, with a series of independent events. The latter possibility became more probable when it was found that variant formation was not eliminated when the size of the population was cut down by very small inoculum doses such as 30 to 40 cells. Since established variants grow in a high percentage of A.SW hosts, preimmunized against strain A tissue, the clonal hypothesis would also imply that the frequency of variants would not decrease if preimmunized A.SW hosts were used for testing the original line. The average frequency of variants decreased in fact from 24 to 2 percent in such an experiment. Taken together, these findings represent indirect evidence favoring the explanation that variants may develop by multiple independent events.

More direct evidence to the same effect could be obtained from the analysis of the detailed karyologic picture of 2 sublines of the original tumor and 10 different variants obtained from 10 individual A.SW hosts. The 10 donor mice had received inoculums derived from 7 different pools of tumor material representing different transfer generations of the original MSWB sarcoma. Four variants arose from 4 different pools, while the remaining 3 inoculums gave rise to 2 variants each. Eight of the variants obtained were all different from the original line and from each other with regard to the detailed chromosomal picture of their stemline cells, irrespective of their derivation from different or common pools of tumor material. On the other hand, two variants (V_{1a} and V_{1b}) were completely indistinguishable from each other, though different from the original line and all other variants. The differences between the different karyotypes were reflected by different stemline numbers and the appearance or the disappearance of distinctive marker chromosomes.

The original MSWB line itself, which was carried in (A × A.SW)F_1 hybrids, remained highly constant with regard to the chromosomal constitution of its stem line, in spite of the fact that 2 different sublines had been carried independently for 7 and 11 transfer generations, respectively. Variation around the stem line was considerably reduced by serial passage in both sublines. In several instances variants were carried serially in A.SW mice, but nevertheless they maintained their characteristic chromosomal features in a highly stable fashion. One variant, V_2, was returned to the original (A × A.SW)F_1 hybrid type in which it was maintained for 15 serial passages without any further change in its chromosomal constitution or its transplantation characteristics.

The evidence thus obtained seems to warrant the conclusion that variants appear repeatedly in the MSWB tumor by multiple independent events. Passage through A.SW mice does not seem to be a strictly necessary condition for the appearance of the variants; the occurrence of 2 variants with identical karyotypes in 2 different A.SW mice inoculated from the same pool shows that a variant clone already pre-existed in the original tumor, in this particular case, though this line had never been outside the (A × A.SW)F_1 hybrid genotype.

A similar combination of multiple independent variation and persistence of one variant clone in a tumor-cell population was also observed in some as yet unpublished experiments on the MACC sarcoma, a tumor induced in an (A × A.CA)F_1 hybrid. As described elsewhere (*19*), this tumor tended to give rise to a certain proportion of variants in both parental strains, though showing preference for the maternal A.CA strain. Subsequent to the selection of one particular variant capable of growth in the paternal A strain and distinctly different from the original tumor stem line, cytologic examination of the unselected population revealed the appearance of the same karyotype, reaching the frequency of about 30 percent, thus producing a mixed population. Concomitantly with this cytologic finding the transplantation behavior of the original line showed a corresponding change as it became able to grow in nearly 100 percent of strain A mice instead of 29 percent as previously. These observations are consistent with the appearance and establishment of a variant clone in the original, unselected population. In contrast, another variant selected in a maternal A.CA strain mouse showed an entirely different karyotype that could not be detected in the original unselected line. This variant must have arisen by another independent event either before or after the test inoculation.

The serologic analyses performed in the present study have demonstrated the existence of the *H-2*-determined isoantigenic components D and K (derived from strain A), S and G (derived from A.SW), and E and F (common to A and A.SW) in the original line of MSWB. All variants studied showed the loss of detectable D and K, while S, G, E, and F were still present. One exception is represented by V_6 [selected in an (A.SW × DBA/2)F_1 hybrid] which has not been analyzed serologically as yet, but whose transplantation data indicate the loss of K only.

There is no conclusive evidence which would prove that the loss of D and K from most variants represents a change in the genetic determinants; in fact, the change might occur at any level. It may be pointed out, however, that the permanence of the change and the highly selective compatibility of the established variants is at least not incongruous with a genetic change. If this were the case, the simultaneous loss of D and K could be postulated to reflect a change more extensive than a single point mutation, since crossing over has been indicated between D and K (*17*). This is not in disharmony with the cytologic findings which show the occurrence of extensive chromosomal rearrangements in the variants. These rearrangements might involve the *H-2*-bearing chromosome region directly. Alternatively, the change might be a more indirect result of the remodeling of the genetic background resulting in changes of modulators, suppressors, position-effects, etc.

It may be appropriate to comment on the fact that no variants have ever been obtained in the paternal A strain. This is not an isolated finding. Studies were made on a larger material, which included 23 tumors of 5 different F_1 hybrid genotypes (*19*). It was found that the maternal strain is the regularly preferred site of variant formation, and whenever paternal variants are formed, they are less compatible with their new host strain than the maternal variants. There are several conceivable interpretations that may explain this finding. The most tempting hypothesis would postulate the presence of some self-reproducing extrachromosomal particles derived from the mother and transmitted to the tumor cells. This particle would have a certain isoantigenic activity, when tested in the paternal strain, thereby preventing the appearance of paternal variants to a large extent. The evidence for the existence of such a maternal effect is not entirely conclusive, however, since tests on the reciprocal hybrid types are not yet available. It must also be taken into account that differences in strength have been demonstrated between the different isoantigenic barriers that result from allelic variation within the *H-2* system (*14*). This may be of relevance in the present context. When MSWB tumors were inoculated into mice of both parental strains and the inoculation site was examined daily, it was found that very little growth occurs in the A strain and all grafts undergo rapid and complete breakdown. Mitotic figures are very scarce, but when they occur they correspond to the MSWB karyotype. In contrast, there is regularly a great deal of background growth in the A.SW hosts preceding the appearance of regressive changes. During the first 8 days after inoculation, mitoses were frequently found and they showed the typical MSWB karyotype. After the 8th day regressive changes started to appear and concomitantly there was an increased chromosomal variability in the metaphase figures. Mitoses gradually disappeared in most cases, but in one instance the appearance of an entirely new stem line could be found 23 days after inoculation. Background growth previous to regression may thus be an important factor that may favor the appearance of compatible variants before the intensification of the selective pressure. The importance of such back-

ground growth is also suggested by the finding that variant formation in A.SW mice drops from 24 to 2 percent if the homograft reaction is accelerated by previous immunization.

Finally, in view of Hauschka's and Levan's findings (*4*, *5*) with regard to correlations between increased chromosome numbers and reduced transplantation specificity in certain tumors, it may be of interest to point out that the only variant that showed a certain tendency to grow in foreign genotypes (V_2) was also the only one that had its main stem line in the near-tetraploid region.

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PLATES

PLATE 83

FIGURE 1, A *to* I.—Metaphases of MSWB, V_{1a} and V_{1b}, V_2, V_3, and V_{4a}. A, B, and C: Metaphases of MSWB stemline $_1$ cells with typical (A) and atypical (B, C) chromosome constitution. A: Metaphase of MSWB s_1, 1 chromosome with a secondary constriction; 41 chromosomes. B: Metaphase with 2 markers; 41 chromosomes. C: Metaphase with 1 marker and 1 metacentric chromosome; 40 chromosomes. D: Metaphase of a V_{1a} stemline $_1$ cell, 1 long chromosome with a secondary constriction and 3 minutes (M); 47 chromosomes. E, F, and G: Metaphases of V_2. E: Stemline $_3$ cell with 1 M; 44 chromosomes. F: Stemline cell with 2 M; 54 chromosomes. G: Stemline $_1$ cell with 4 M; 85 chromosomes. H: Metaphase of an s_1 cell of V_3, 1 metacentric marker and 1 chromosome with a secondary constriction. I: Metaphase of a V_{4a} stemline $_1$ cell; 40 chromosomes. × 1,150

A
B
C
D
10 μ
E
F
G
H
I

PLATE 84

FIGURE 2, A *to* F.—Metaphase of V_{4b}, V_{5a}, V_{5b}, V_6, and V_7. A: Metaphase of a V_{4b} s_1 cell, 1 long subtelocentric chromosome with a secondary constriction and 1 minute (M); 42 chromosomes. B: Stemline $_1$ metaphase of V_{5a} with an acrocentric marker (proportion of the arms about 1 : 3) and a long chromosome with a secondary constriction in the middle of the long arm. C: Stemline $_1$ metaphase of V_{5b}, 1 long acrocentric chromosome, arm-length proportions about 1 : 3, absolute length greater than that of the acrocentric chromosome of V_{5a}, and a chromosome with a secondary constriction in the long arm. D: Metaphase of V_6s_1, 3 metacentric chromosomes and 1 chromosome with a secondary constriction. E: Metaphase of V_7s_1, 2 metacentric chromosomes and 1 chromosome with a secondary constriction. F: Metaphase of a V_7s_2 cell, 1 superlong subtelocentric chromosome, 3 metacentric chromosomes, and 3 chromosomes with secondary constrictions. × 1,150

A
B
C
10 μ
D
E
F

PLATE 85

FIGURE 3.—Idiograms of the chromosomes of MSWB and the variants. Based on drawings and measurements of the chromosomes of 6 metaphases with the stemline $_1$ modal number and the typical marker-chromosome equipment for each tumor. The 6 metaphases were derived from the same transfer generation for each tumor. × 750

Bayreuther and Klein

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The RPL12 Lymphomatosis Virus in Chicken Embryo and in Chicken-Embryo Tissue Culture. Morphological and Cytochemical Observations[1]

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Summary

The alterations induced by the RPL12 lymphomatosis virus in chicken-embryo liver tissue culture and in chicken embryo were described. In the tissue-culture system, large intranuclear inclusions leading to eventual cell destruction were observed; the number of inclusions and the time necessary for their formation were related to the amount of virus introduced. Cytochemical studies indicated that the inclusions were rich in deoxyribonucleic acid. At the terminal stage the inclusions consisted of a dense central body surrounded by a chromophobe halo and an external ring of basophilic material. The evolution and morphology of these inclusions seemed to be fairly characteristic for this virus. In the 12-day-old chicken embryo, undiluted virus preparations regularly induced a series of changes: *Period I* (*0 to 48 hours*)—Intranuclear inclusions formed in the hepatic cells, with morphological and cytochemical characteristics similar to those observed in tissue culture. *Period II* (*48 to 72 hours*)—Large basophilic cells proliferated in the mesenchyma of the liver and other organs. Cells with inclusions were still present. On the basis of its characteristics, the proliferative process has been considered similar to the initial phase of the experimental lymphomatosis in the adult bird. *Period III* (*72 hours to hatching time*)—Proliferative lesions persisted in the liver and were accompanied by large areas of necrosis. The relationship of the virus to the characteristic cytolytic and proliferative changes was discussed and some evidence was brought forward for an interpretation of the two processes as an expression of the same virus.—**J. Nat. Cancer Inst. 21: 925–959, 1958.**

Recent reports (*1*, *2*) have described the successful growth of avian lymphomatosis virus, strain RPL12, in tissue cultures of whole chicken embryo and chicken embryo liver. The virus grown in these systems has a distinct cytopathogenic effect, produces characteristic lesions when inoculated into the chicken embryo, and induces lymphomatosis when inoculated into chicks.

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The RPL12 is a transplantable tumor (*3*) from which a filtrable agent has been isolated (*4*) that induces a high incidence of tumors in susceptible birds (*5*). The virus was passed in the chicken embryo for the first time by Gentry and Burmester (*6*) who demonstrated actual growth of the virus without associated macroscopic lesions. Brandly *et al.* (*7*) transplanted 2 strains of lymphomatosis tumor cells in embryos. The tumors could not be passed serially, but a leukemoid response was observed, which lacked specificity since it was also provoked by tissues of apparently normal birds and by blood of birds infected with erythrogranuloblastosis virus. Pathological changes in chicken embryo have been described and correlated with inoculation of other viruses of the leukosis complex and of Rous sarcoma. Milford and Duran-Reynals (*8*) reported hemorrhagic lesions after inoculation of the Rous sarcoma virus; Hall *et al.* (*9*) propagated erythroblastosis in the embryo by intravenous inoculation, and Atanasiu (*10*) found a myeloblastic response in the blood of embryos inoculated with erythroblastosis virus.

While growth of the lymphomatosis virus in a tissue-culture system has not been previously reported, the maintenance and growth *in vitro* of viruses of the leukosis group and of related Rous sarcoma, either in cultures of the originally infected cells (*11–13*) or of chicken fibroblasts (*14*), have been described. No specific alterations were found in any of these cases except for cellular degeneration and necrosis in chicken fibroblasts infected with the Rous sarcoma (*15*, *16*).

It is the purpose of this paper to describe and analyze the pathological changes induced by the RPL12 lymphomatosis virus in chicken-embryo liver tissue culture and in the chicken embryo. The specificity of these changes as compared to the effect of other viruses will be discussed as well as the possibility of a cytolytic and oncogenic phase during infection by the virus.

Materials and Methods

Virus.—The virus, designated as strain RPL12, passage $L28_1$, was originally received from Dr. B. R. Burmester (Regional Poultry Research Laboratory, East Lansing, Michigan) as a lymphomatous liver filtrate, representing its sixteenth serial passage in White Leghorn chickens. In the experiments herein described, the thirteenth and fourteenth passages in chicken-embryo liver tissue culture (*1*) were utilized. The 50 percent tissue-culture infectious doses (TCID50) of these 2 virus pools were $10^{8.5}$ and $10^{9.5}$ per ml., respectively.

Tissue culture.—The cultures, obtained from trypsinized suspensions of 15-day-old chicken embryo liver, were grown on coverslips in Leighton tubes. Each tube received approximately 500,000 cells in 1.0 ml. of medium composed of 30 percent horse serum and 70 percent medium 199 (*17*). The cultures were incubated for 48 hours at 37° C., allowing the formation of a cell monolayer; the medium was then replaced with 100 percent medium 199, and the cultures were inoculated with the infectious filtrate.

In a typical experiment, tubes were inoculated with progressive tenfold dilutions of the virus, from 0 to 10^{-5}, and 2 samples from each dilution were taken for cytological observation at 3, 6, 9, 18, 24, 30, 36, 50, 74, and 86 hours. In this way it was possible to observe and evaluate the progress of the lesions induced as a function of the virus titer and the duration of infection. Control tissue cultures were inoculated with supernatant fluid from uninfected tissue cultures.

For routine examination the cultures on coverslips were fixed in Bouin's fluid for 10 minutes and stained with hematoxylin and eosin. Other fixatives were used for cytochemical investigation as indicated in the following sections.

Chicken embryo.—Twelve-day-old chicken embryos were used in all experiments. Inoculation was made in a vein of the chorio-allantoic membrane with a 27-gauge, ½-inch needle after the membrane had been exposed and the field clarified with mineral oil. The window in the shell was then sealed with Scotch tape and the egg replaced in the incubator. In each experiment 3 groups of embryos were utilized. The totals for all experiments were as follows: 130 embryos received infectious supernatant fluid, 28 were inoculated with fluid from noninfected tissue culture, and 20 were not inoculated. Each inoculated embryo received 0.10 ml. of fluid. Virus-inoculated and control embryos were sacrificed approximately every 6 to 12 hours. Mortality in the virus-infected groups was very high from the 3rd to 4th days after inoculation, and only a few specimens were available for study at hatching time.

At autopsy, blood smears were obtained from vessels of the chorio-allantoic membrane and the heart cavities. Histological studies were made of specimens consisting either of the whole embryo or of parts of the following organs: chorio-allantoic membrane, yolk sac, heart, lung, liver, kidney, stomach, small intestine, spleen, bursa of Fabricius, and femur. Imprints were also made from the femoral bone marrow. The specimens were immediately fixed in Bouin's fluid or formol-sublimate solution. In some instances, the whole embryo was fixed in Bouin's fluid, and, beginning at the right paravertebral line, sagittal sections were serially cut through the left side of the body.

Cytochemical techniques.—Deoxyribonucleic acid (DNA) was stained by the Feulgen technique with N HCl hydrolysis for 8 minutes at 60° C. (*18*); occasionally azure A-Schiff reagent was used in place of the basic fuchsin (*19*) with similar results. Control sections were incubated at 37° C. for 2 hours with 0.1 percent DNase[4] in a solution of 0.003 M $MgSO_4$. Ribonucleic acid (RNA) was demonstrated by the methyl green-pyronin method (*18*) in conjunction with RNase[4] treatment (0.1% at 37° C. for 1 hour) of control sections. In some cases, both nucleic acids were extracted with 5 percent trichloroacetic acid at 90° C. for 15 minutes.

For the study of cellular proteins the following methods were employed: the alkaline-fast-green method of Alfert and Geschwind for nuclear basic proteins of the histone type (*20*); the ninhydrin-Schiff reaction for

[4] Obtained from Worthington Biochemical Co., Freehold, N. J.

primary amino groups (*21*); the tetrazonium reaction (*18*); the methylene-blue extinction (*18*) on a *p*H range from 2.8 to 6.5; and the Barrnett and Seligman method (*22*) for SS and SH groups. The periodic acid-Schiff reaction (*18*) was used for the study of 1-2 glycols in conjunction with diastase[5] digestion for demonstration of glycogen. Lipides were stained with Sudan black B and Nile blue (*18*). Alkaline and acid phosphatases were studied with the coupling azo-dye technique (*18*). In addition, mitochondria were stained with Heidenhain's iron hematoxylin (*23*); this stain was also useful for the demonstration of inclusion bodies even after prolonged differentiation.

Results

Tissue Culture

The chicken-embryo liver tissue culture, when grown as a monolayer by the method described, consisted of discrete islets of hepatic cells surrounded by interlacing bands of fibroblasts. In the usual preparation there was an average of 40 to 100 parenchymal cells in each island. The parenchymal cells in the intermitotic phase exhibited tinctorial and cytochemical properties similar to those of the tissue of origin. Ribonucleic acid was fairly abundant in the cytoplasm and in the large nucleolus, and deoxyribonucleic acid (fig. 1) was distributed throughout the nuclei in a fine reticulum with scattered chromatin granules and in a narrow rim around the nucleolus. The cells were rich in glycogen and contained a few large droplets, mostly neutral lipides, and numerous fine granules of acidic lipides and protein complexes. With aging of the cultures there was a noticeable increase in the number and size of the large droplets. Mitochondria were rather numerous and round. No alkaline phosphatase was demonstrated by the technique used, while a moderate reaction for acid phosphatase was observed in the cytoplasm.

The sequence of changes in cultures inoculated with infectious suspensions was more easily demonstrated when an undiluted filtrate was used. Under this condition almost all the hepatic cells appeared to be infected, and the alterations followed a contemporaneous course in all the cells. Inoculum dilutions of 10^{-2} or 10^{-3} were found more useful in following specific changes over a prolonged period.

The changes occurring in tissue cultures infected with filtrates diluted 10^{-1} are reported here as a typical experiment: The earliest appreciable changes began to appear toward the 18th hour after infection. At this time the hepatic cells were generally enlarged and showed increased granularity of the cytoplasm. The nucleus was denser and there was occasional margination of the chromatin; soon thereafter the nucleolus, often swollen, was pushed to the periphery close to the nuclear membrane. Small blocks or filaments of hematoxylinophilic material, distinct from the chromatin reticulum, were present in the nucleus. These new basophilic formations were characterized by a very wide polymorphism, as illustrated

[5] Obtained from Merck & Co., Inc., Rahway, N. J.

in figures 4, 5, and 6. They varied from uniformly dense blocks to ringlike formations containing either a slightly acidophilic material or basophilic bodies. There was no constant pattern in the localization or number of these formations; at times they were in the center of the nucleus, sometimes in proximity to the nuclear membrane, and at other times, alone, almost completely filling the nucleoplasm. All the infected nuclei had diameters 2 to 3 times larger than those of the uninfected cells, and in hematoxylin and eosin preparations the cytoplasm had a diffuse, glassy appearance. The nuclear membrane became progressively less distinct, and the terminal phase of the process was reached about 24 to 30 hours after infection. It was characteristic that the basophilic formations appeared multiform in the initial stages and fairly uniform in the terminal phase. They consisted of a central, very dense, round or ovoid body, 8 to 10 microns in diameter, surrounded by a chromophobe halo and by an additional external ring of basophilic material (fig. 7). In the chromophobe zone, hematoxylinophilic filaments radiating from the central body were often observed, together with granules or fragments. Occasionally a honeycomb structure of the central body (fig. 10) was noticeable. These formations were visible for 10 to 12 hours, after which there was a complete breakdown and loss of the hepatic cells. Although no significant changes were observed in the surrounding fibroblasts during this period, when the destruction of the hepatic islets occurred, the fibroblast nuclei assumed a tigroid aspect with numerous small hematoxylinophilic spheres having hollow centers or with large basophilic blocks. Observation beyond this stage was usually prevented because of the separation of the monolayer from the surface of the coverslips.

The Feulgen reaction showed that in the infected nuclei there was a tremendous increase in DNA; its localization in the initial phase was limited to filamentous or granular bodies of the type previously described (figs. 2 and 3). In the large bodies occupying the whole nucleoplasm, the increase was diffuse with particular concentration in small blocks. The distribution of DNA in the terminal phase of the infection was also rather characteristic. The large central block and the peripheral fragments were strongly and uniformly positive, and the chromophobe halo showed a moderate reaction (figs. 8 and 9). No evidence of DNA material or particles was seen in the cytoplasm. The methyl green-pyronin method confirmed the initial and short-lived swelling of the nucleolus with an increased pyroninophilia of the cytoplasm. The pyroninophilia lasted until the terminal phase. With the formation of the large central body, the nucleolus lost its morphological characteristics, but a strongly pyroninophilic formation, most often in a half-moon shape, was frequently seen at the periphery of the nucleus (fig. 13). It is not clear whether this formation represents the original nucleolus, deformed and pushed to the periphery, or the nucleolar RNA rearranged and redistributed. Later, this body was no longer evident.

At the beginning of inclusion formation, the alkaline-fast-green method for basic protein did not show any changes in the amount of bound dye.

In the final stage, the central body was only partially stained, but the fragments at the periphery were a brilliant green (fig. 12). The large, round inclusions gave a strong reaction with ninhydrin-Schiff and tetrazonium and showed a light reaction for sulfhydryl and disulfide groups. There were no alterations in the carbohydrate components of the cells, except for a progressive loss of glycogen. The lipide stain demonstrated an increased uptake of dye by the infected cells in the juxtanuclear area (fig. 11), possibly associated with the components of the Golgi apparatus. This fact was observed both with Sudan black B and Nile blue; with the latter dye, the increase was at the level of the neutral fats as well as of the acidic lipides. With the progress of the alteration, there was a general increase in the neutral fat components and a disappearance of the acidic lipides. Large and numerous droplets of neutral fat were seen in the final degenerative stage. No particular changes were observed in the alkaline and acid phosphatases.

Chicken Embryo

The mortality in the 12-day-old chicken embryos inoculated with infectious material varied in different experiments depending upon the concentration of virus employed. When high titers of virus were used in the inoculum, most of the embryos died on the 3rd and 4th day after infection. In embryos inoculated with noninfectious material the mortality was high, approximately 13 percent (5 out of 38 and 2 out of 17 in 2 separate experiments) on the 1st day after inoculation, with no deaths on the 2nd through the 7th day.

At gross examination, the alterations in the embryos infected with the virus were not striking. However, in almost all the infected embryos, beginning on the 3rd day after inoculation, yellowish, depressed areas were noted on the surface of the liver lobes, usually toward the anterior edge. Similar areas of discoloration, surrounded by hyperemic zones, were observed on the cut surface of liver cross sections. Embryo livers normally go through a process of increased lipide content that manifests itself by the appearance of yellow spots about the 12th to the 15th day and by a diffuse sulfur-yellow color at hatching time (*24*) as was seen in the control embryos. It is felt, however, that in view of the correlated microscopic findings, the discoloration seen in the infected embryos was of a different nature. In addition, in virus-infected birds the spleen was often enlarged, firmer in consistency, and the weight usually higher than, or in the upper limits of, the weight range of the spleens from the 2 control groups. No other abnormalities were observed in the body cavities, other organs, or embryonic membranes. Of 130 virus-infected embryos that were autopsied, 2 specimens showed congenital malformations: one, a 13-day-old embryo, did not have eyes, forebrain, or upper beak; the other, also 13 days old, had brachimelia of the wings, reduction of the length of the vertebral column, and deformation of the pelvis.

In contrast to the paucity of gross lesions resulting from virus infection, the microscopic examination revealed a series of changes that can be

described and classified in relation to their form and time of appearance. Roughly, three periods could be distinguished: the first, from 0 to 48 hours after inoculation, in which the lesions were localized in the hepatic cells and were essentially characterized by the formation of intranuclear inclusions; the second, from 48 to 72 hours after inoculation, in which proliferative changes in the liver and mesenchyma of other organs and cells containing intranuclear inclusions were recognized; and the third, from 72 hours after inoculation until hatching time, in which only proliferative lesions and large areas of necrosis were present in the liver. The distribution in time of these alterations is given in table 1.

I. *First period; 0 to 48 hours after inoculation.*—The development and characteristics of the inclusion bodies were essentially similar to those previously described in the hepatic cells of the liver tissue-culture system. In some hepatic cells, large and swollen nuclei containing numerous and conspicuous bodies appeared (fig. 14), accompanied by an increased density of the cytoplasm with swelling of the mitochondria (fig. 17). The inclusion bodies, though not so multiform as in the first phase of the tissue-culture infection, still showed considerable variation in size and shape with a high frequency of large basophilic blocks. In a more advanced stage, the nuclei of the affected cells appeared as large sacs that contained a uniform basophilic sap, punctuated by denser aggregates of basophilic material (fig. 15). The number of cells showing these alterations was variable from one specimen to another—1 to 10 in each high-power field. In some circumstances there was a suggestion of an accumulation of affected cells near the central vein, but a definite pattern of distribution could not be established.

The cytochemical characteristics of the cells were also similar to those in tissue culture. The nuclear sap and the aggregates were strongly positive with the Feulgen reaction (fig. 16); light staining was observed with the fast green, occasionally more intense in the small blocks. The latter were strongly positive with the tetrazonium reaction (fig. 18), while the rest of the nucleus was negative. By the methylene-blue extinction method, it appeared that the inclusions lost their capacity of binding any dye below *p*H 5.2, demonstrating the absence of acid mucopolysaccharides. No lipides or carbohydrate groups were shown in the nucleus. In the cytoplasm of these cells, a transient increase of pyroninophilia was found that was abolished by ribonuclease. The fate of the nucleolus was not clear, since only rarely could pyroninophilic material be observed in the nucleus and in such cases it was pushed to the periphery of the nucleus in a sickle shape. The intranuclear material reacted slightly with the test for sulfhydryl and sulfide groups and there were no changes in the amount and distribution of the latter in the cytoplasm. Acid phosphatase was positive in the affected as well as in the unaffected cells, and alkaline phosphatase was limited to the biliary capillaries and ducts.

II. *Second period; 48 to 72 hours after inoculation.*—At the end of the 2nd day after inoculation, while cells with nuclear inclusions were still present, a different type of cells appeared in the portal spaces of some of

TABLE 1.—*Twelve-day chicken embryos inoculated intravenously with the 13th tissue-culture passage of RPL12-L28 (data pooled from 3 experiments)*

Time from inoculation (*hours*)	Number of embryos		Type of hepatic lesions		
	Examined	With hepatic lesions	I Inclusion bodies	II Necrosis	III Proliferation
4	2	0	0	0	0
8	2	0	0	0	0
14	2	1	1	0	0
24	2	2	2	0	0
	3*	2	2	0	0
28	2	2	2	0	0
32	2	2	2	0	2
38	2	2	2	0	2
48	6	4	4	1	3
	5*	4	4	1	2
72	9	7	3	4	7
	18*†				
96	2	2	0	2	2
144	4	2	0	1	2
168	4	1	0	1	1
	4*	2	0	2	2
192	2*	0	0	0	0

*These embryos found dead.
†In many of these embryos *post-mortem* autolysis was too far advanced to allow any precise histological evaluation.

the embryos: They were round, larger than the contiguous hepatic cells, with an intense basophilic cytoplasm and a round. conspicuous, clear nucleus that contained a well-defined, nucleolus-like structure (fig. 24). These cells were quite distinct from the cells which at this time (14 to 15 days) began to appear in the same location in the normal embryo and that were for the most part heterophilic leukocytes with some lymphoblasts, as seen in our controls and as reported by Kingsbury *et al.* (*26*). In the beginning, the abnormal cells were scattered in the perivascular space (figs. 20 and 21); at a later period they became very numerous, forming a dense perivascular cuff (fig. 23). Localization in the portal spaces was most frequent, though groups of such cells could be seen occasionally around the central vein or as isolated units between the hepatic tubules; in the latter case, they were very bulging and occluded the tenuous hepatic sinusoids. While most of the cells were extravascular, isolated instances were encountered of cells lying just across the vessel's wall. The presence of these cells was observed, not only in the liver but in the loose mesenchyma of the myocardium (fig. 22), mesonephron, and metanephron.

With the appearance of abnormal cells, many large areas of necrosis were seen in the liver. They were usually found toward the edge of the organ and, in some cases, a characteristic chessboard pattern was observed (fig. 25) with the necrosis around the central vein and the normal parenchyma around the portal spaces. In most cases, the large, round cells formed a thick cuff around the vessels in the portal area. No inflammatory reaction was seen, only dilation and stasis in the contiguous sinusoids.

The changes in the spleen can be better appreciated by comparison with the spleens of control embryos. At this stage (14- to 15-day-old embryo) the spleen normally presents a complex picture since hematopoietic activity has begun; thus, primitive reticular cells are encountered, as well as myelocytes and heterophilic leukocytes, lymphoblasts, lymphocytes, and some erythroblasts (fig. 27). There is no differentiation of follicles and red pulp as yet; this is apparent toward the 18th or 19th day. In contrast, the spleens of the infected embryos presented a very uniform field, which consisted of large, compact cells with voluminous nuclei, somewhat resembling the cells observed in the mesenchyma of the liver and other organs, and primitive reticular cells (fig. 28). No other cellular elements indicating any orderly hematopoietic activity were encountered. The changes in the spleen were observed only when alterations of the type previously described occurred in the liver.

III. *Third period; 72 hours after inoculation until hatching time.*—In embryos of this age, the lesions were characterized as follows: In the liver, no cells with intranuclear alterations were observed; the proliferative lesions in the portal spaces were more advanced than in the previous stage, with some evidence of transformation of the large, round cells into cells with fibroblastic characteristics. In two instances, there was actually an area of complete fibroblast replacement in one portal space, accompanied by proliferation of the biliary ducts (fig. 26). The fibroblasts

infiltrated through the hepatic tubules and there were no definite borders between the two tissues. Necrosis of the parenchyma always accompanied such lesions. In the spleen, the uniform pattern was again observed, with complete absence of any hematopoietic activity throughout the entire period.

Examination of other organs of infected embryos did not show any deviation from the normal as represented by the control embryos. Differential counts of blood smears obtained from heart cavities and yolk sac did not show any differences and no abnormal cells were seen. The progressive replacement of embryonic erythrocytes by mature ones followed a normal pattern. Virus could be recovered from the liver of infected embryos any day after inoculation.

The changes described were observed regularly in embryos inoculated with undiluted virus preparation and sporadically in those inoculated with 10^{-1} and 10^{-2} dilutions. No significant alterations were observed in the 2 control groups. Only 2 embryos inoculated with noninfectious material showed areas of liver necrosis, and these were small.

Discussion

The changes induced by the lymphomatosis virus in the nuclei of hepatic cells, either in tissue culture or in the chicken embryo, correspond to the alterations that in recent years have been associated with the proliferation of viral agents (*25–29*). Briefly, they consist of an enlarged nucleus, a loss of identity of the original chromatin of the cell, and a progressive increase in DNA that is present in the nucleoplasm either in diffuse form or in small blocks. The final stage of the inclusion is very similar to the flowerlike nucleus described in HeLa cells after infection with type 3 and 4 adenoviruses (*28*) or to the inclusions induced by the polyhedral virus in the silkworm (*26*).

The evolution of the lymphomatosis inclusion, however, presents some unique characteristics: The increase in DNA is observed during all stages of development with no acidophilic body as there is in the initial stage of the infectious papilloma of the human skin (*30*) or in the terminal phase of the herpes simplex inclusions (*25*). Furthermore, the lack of increase in staining with fast green, in spite of the Feulgen increase, has shown that the DNA synthesized in the cell during the virus infection is associated mostly with proteins other than histone or that there have been changes in the histone molecule itself. That some proteins are present in the inclusion bodies is demonstrated by the intense reaction with ninhydrin-Schiff, with tetrazonium, and with the differentiated stain with methylene blue. The only location in which a DNA-histone association has been preserved in seemingly normal proportion is observed in the outer portion of the nucleus containing the large inclusion; there (fig. 12), small fragments reacting intensely with the Feulgen and fast green are found. Whether these fragments represent the residual chromatin redistributed at the nuclear membrane or new units connected with actual virus particles is not clear. It is noteworthy that this distribution is evident only at the terminal phase of the inclusion.

In summary, one can visualize in the nucleus a series of zones or gradients in which DNA and proteins, of which histones represent only a small part, are formed, distributed, and associated in different degrees. Since we do not have data on the quantitative aspect of the virus production, it is not possible to correlate these changes in the nucleus with the actual formation of viral units. Also, the suggestion that the DNA increase represents viral DNA can be supported only on circumstantial evidence—the high titer of the tissue-culture fluid immediately following the terminal phase of the inclusion (*1*)—and by analogy with other animal viruses (*29*). No information is available at this time on whether the lymphomatosis virus is a DNA virus.

The changes in other chemical components of the cell, while often correlated with virus infection, are not specific for fowl lymphomatosis virus; there is a transient increase of the cytoplasmic RNA and an enlargement of the nucleolus in the initial phase, which is observed also with the herpes simplex virus by Crouse *et al.* (*25*) and correlated with a possible protein synthesis stimulation. In the cytoplasm there is a definite increase in both acidic and neutral lipides during the inclusion formation, which is accompanied by swelling of the mitochondria.

It is of particular interest that the lymphomatosis virus produces alterations in the tissue-culture system and in the liver of the chicken embryo that are essentially similar not only in their morphological and cytochemical characteristics but also in their time of appearance and evolutionary phases. The lifespan of the infected cells in both systems is also similar; that is, in the tissue culture after 50 hours the infected hepatic cells are in complete necrosis, and in the liver of the embryo no cells with inclusion bodies are encountered after 72 hours (table 1). It seems that after an initial wave, no new hepatic cells show morphological changes of that type. But, as has been mentioned previously, a new type of cell that does not correspond to any in the coeval control embryo appears toward the 38- to 48-hour period in the mesenchymal areas of the liver and in other organs. These cells actively proliferate, grow compact, and, in an advanced stage, fill almost all the portal spaces. While it is difficult to classify these cells, their general characteristics are those of very primitive elements of the hematopoietic mesenchyma with the potentiality of evolving into fibroblasts. Cells of somewhat similar characteristics have been described by Groupé *et al.* (*31*) in the liver of chicks inoculated intracerebrally with the Rous sarcoma virus. Loomis and Pratt (*32*) have interpreted round, basophilic cells in the subcutaneous tissue of the fowl as one of the tumor-cell types resulting from the transformation of fibroblasts after inoculation with Rous sarcoma virus. The relationship of these round, basophilic cells to the cells observed with the lymphomatosis virus is not clear at this time.

What is the nature of this proliferative cellular reaction, and what relation does it bear to the phenomenon of the nuclear inclusion? The proliferative phenomenon is observed in areas of liver and spleen that normally undergo an active production of blood elements during the last

week of embryonic life; the abnormal cells could then represent stem cells that have failed to differentiate under some direct or indirect action of the virus. But the reaction described here is something more than an inhibition of differentiation, as the cells proliferate vigorously and infiltrate the hepatic tubules or produce enlargement of the spleen. The proliferation is strictly extravascular as no abnormal cells are seen in the blood, and the blood picture with its shifting proportion of different cellular elements is essentially normal. This is reasonably explained by the fact that the yolk sac, bone marrow, and bursa of Fabricius are not altered.

On the basis of the general characteristics and invasive properties of the cells, the process described in the embryo is similar to that of visceral lymphomatosis of the lymphoblastomatosis type as described in the adult bird (*3*, *33*, *34*). The only thing lacking to complete the similarity is a picture of an advanced stage of the disease in the embryo, which so far it has not been possible to obtain. The high mortality of the embryos has not allowed a definite follow-up after the hatching, but experiments along this line are being continued with the use of more diluted preparations of virus. Necrosis of the liver, particularly when very widespread, probably represents one of the more plausible explanations for the death of the embryo. Necrosis of the liver is not by any means a specific reaction to the lymphomatosis virus. It has been described in a variety of conditions (*7*, *35*, *36*) though its frequency and distribution are not clear. We have found two such instances in embryos inoculated with noninfectious material and none in the noninoculated. In their description of the development of the liver in the chicken embryo, Kingsbury *et al.* (*24*) do not mention any such findings. In some of the embryos inoculated with lymphomatosis virus, necrosis follows a pattern of distribution that would indicate a condition of anoxia as the causative factor. The cells proliferating in the portal spaces produce impairment of the blood flow and of the oxygenation of the tissue, and only the hepatic cells in proximity to the portal spaces are able to survive (chessboard pattern). This interpretation does not exclude a possible concomitant toxic action of the virus itself.

The relation between the formation of intranuclear inclusions and cell proliferation can only be a matter of speculation, because observations were made on a series of specimens killed at various times. Table 1 shows the distribution in time of these two lesions. It is apparent that there is a phase of intranuclear inclusions only, a phase of cell proliferation only, and an intermediate phase in which both lesions are present side by side. If both lesions are of viral origin, two hypotheses can be examined: 1) that each lesion is produced by an independent virus, or 2) that one virus infects two types of cells, hepatic and mesenchymal, inducing in one the formation of inclusions and in the other, proliferation. The fact that these results were obtained with the thirteenth and subsequent tissue-culture passages does not support the first hypothesis, nor do the separate experiments with different passages, which showed that the

two types of lesions remained constant as to proportion and time of appearance. Furthermore, the tissue-culture experiments have demonstrated that the lymphomatosis virus can induce the formation of similar inclusions in tissue culture, and such cultures produce lymphomatosis when inoculated in the chicken. Examination of spray preparation of the supernatant fluid of infected tissue culture by means of electron microscopy has revealed the presence of spherical particles of a homogeneous size, 75 to 80 millimicrons in diameter (*37*).

The second hypothesis is, therefore, more likely to be correct. It is not at all clear why the process of inclusion formation exhausts itself after 48 hours and is not repeated in the following period. The time relationship between the presence of inclusions and the proliferation of mesenchymal cells cannot be readily explained on the basis of the data available, except perhaps by assuming that the virus goes through a proliferative phase in the hepatic cells and subsequently passes to the mesenchymal cells. In any event, it seems highly probable that we have here an example of a virus manifesting its cytolytic and oncogenic phases not as a function of the age of the host—as in the case of the Rous sarcoma virus (*8*)—but as a simultaneous or successive phenomenon in the same host. This raises the question of whether the two phases are also represented in the pathogenesis of the experimental and natural disease in the adult bird, or whether they have become characteristics of this particular strain because of the passage in and adaptation to chicken-embryo liver tissue culture.

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PLATE 86

Figures 1 to 13 are photographs of chicken-embryo liver tissue culture.

FIGURE 1.—Noninfected liver cells. *Note* reticulum arrangement of the DNA. In some nuclei the nucleolus associated chromatin is very evident. Feulgen reaction. × 1,170

FIGURE 2.—Liver cells, 18 hours after infection. The increase in DNA is already manifest in the shape of filamentous or granular bodies, occasionally in a basket-like arrangement. Feulgen reaction. × 1,170

FIGURE 3.—Same as figure 2.

FIGURE 4.—Liver cells, 18 hours after infection; enlarged nuclei with appearance of sickle-shaped, ringlike formations or dense blocks inside the nuclei. Iron hematoxylin. × 1,170

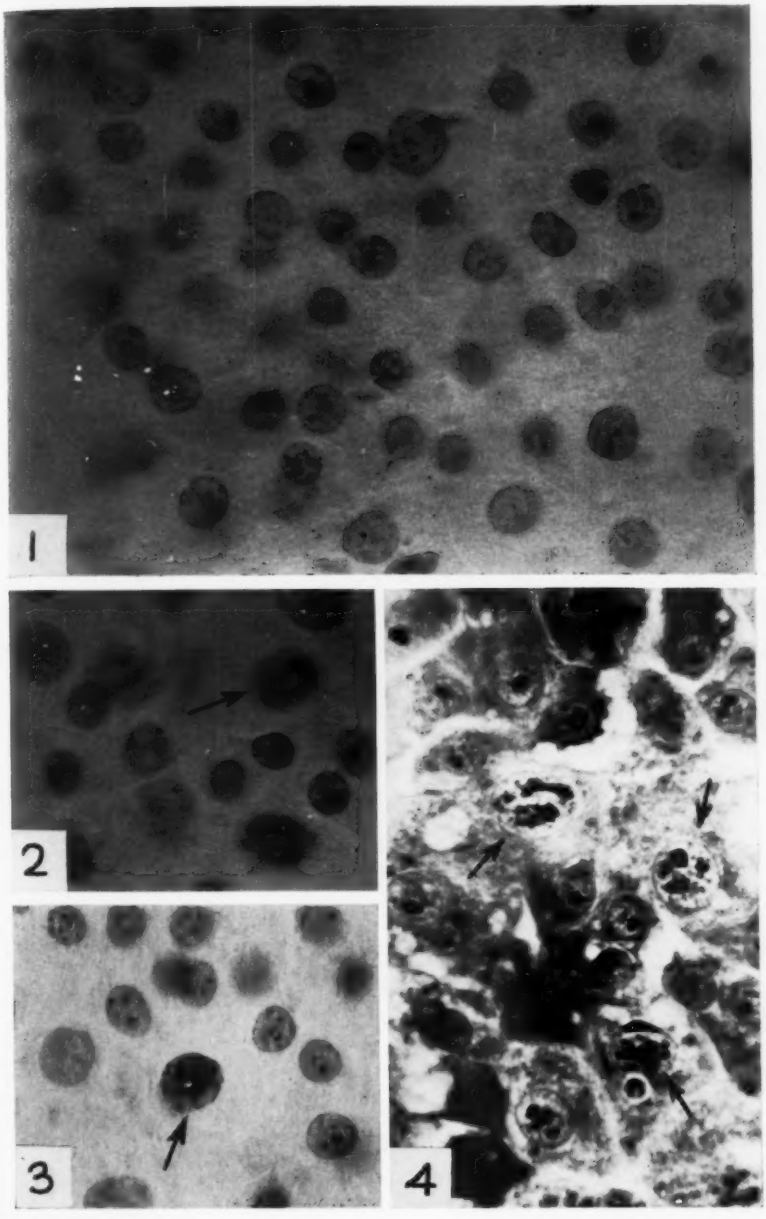

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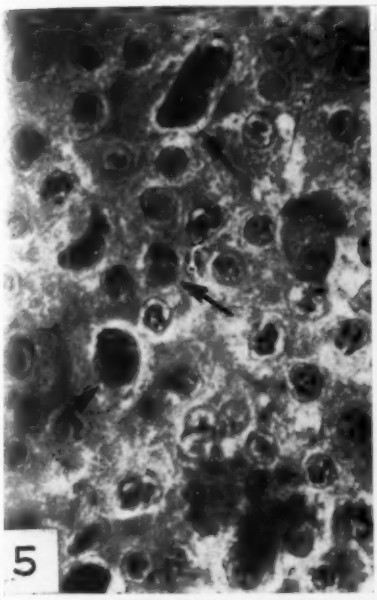
PLATE 87

FIGURE 5.—Liver cells, 24 hours after infection. The polymorphism of the intranuclear formation is clearly evident. Iron hematoxylin. × 850

FIGURE 6.—Same as figure 5. × 1,170

FIGURE 7.—Liver cells, 36 hours after infection. Many cells show greatly enlarged nuclei with intranuclear formations, uniformly dense or having a dense central body surrounded by a chromophobe halo and an additional external ring of basophilic material. Iron hematoxylin. × 850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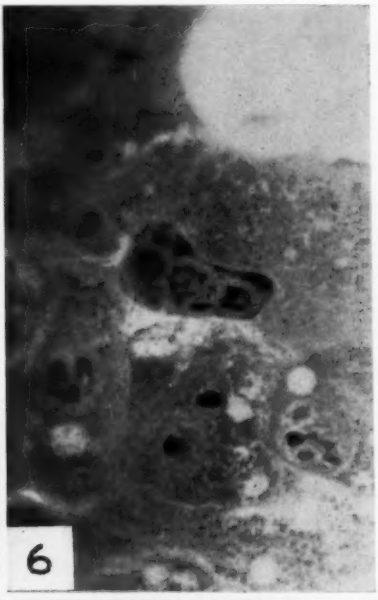
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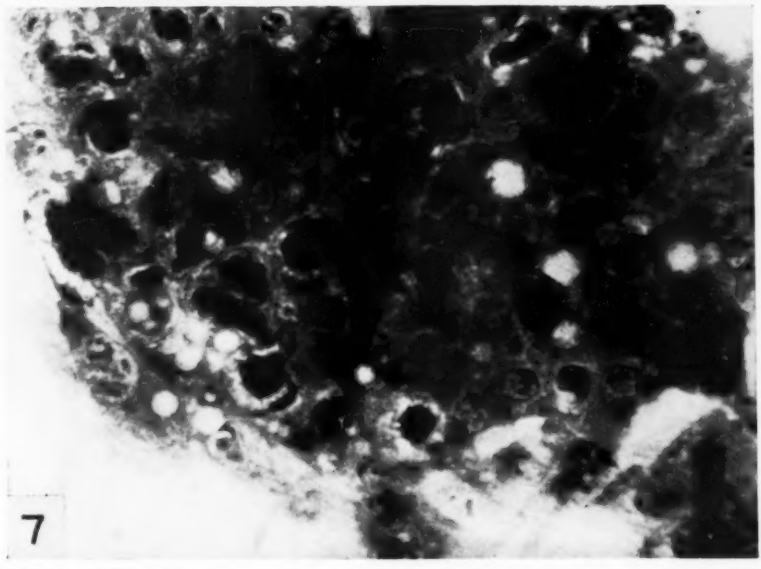
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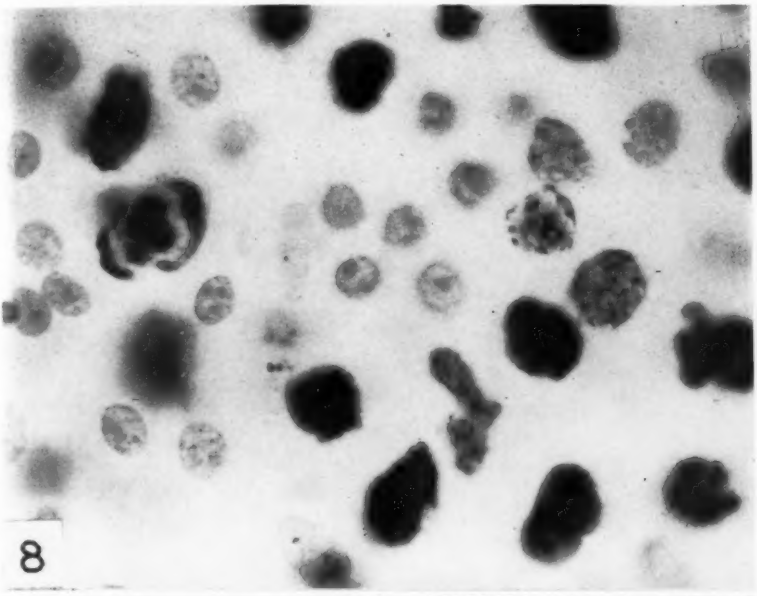
PLATE 88

FIGURE 8.—Liver cells, 36 hours after infection. The DNA in the infected cells is greatly increased. The central body and peripheral ring are intensely positive; in addition, small fragments of DNA material are observed at the nuclear periphery. In *right half* of figure, some nuclei show alterations similar to those illustrated in figures 2 and 3, probably representing a new cycle of infection. Feulgen reaction. × 1,170

FIGURE 9.—Same as figure 8.

FIGURE 10.—Liver cells, 48 hours after infection. Terminal phase of the intranuclear inclusion. Honeycomb structure of the central body is seen with fragments of material in the chromophobe halo and at the nuclear membrane. Iron hematoxylin. × 1,800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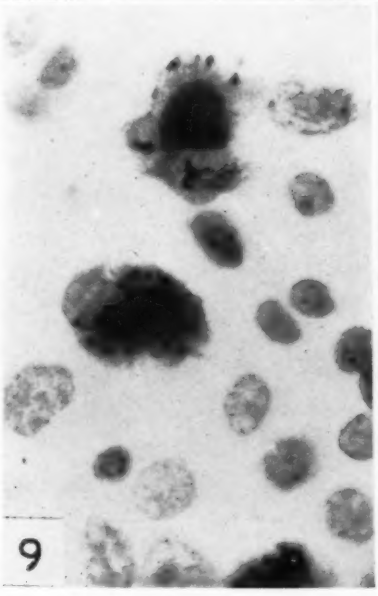
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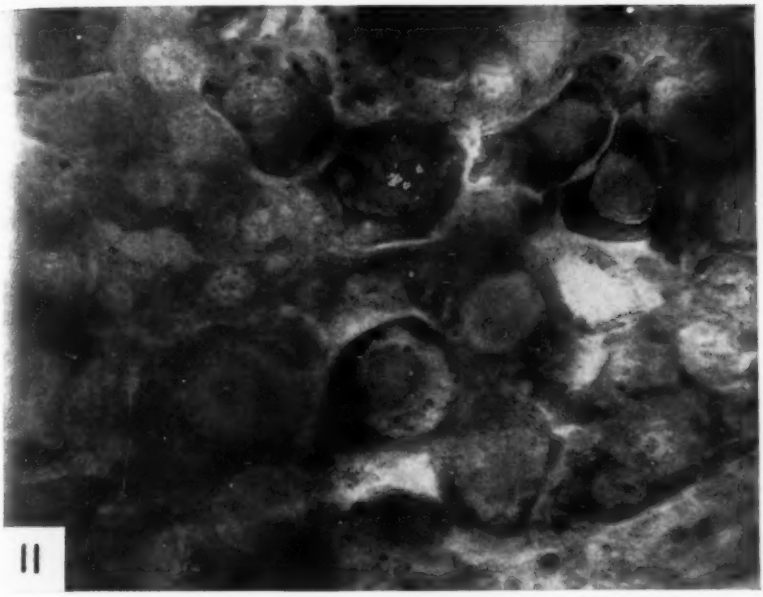
PLATE 89

FIGURE 11.—Liver cells, 48 hours after infection. The dark perinuclear zone of the cells at the center of the field represents the increase of the acidic and neutral lipide components. Intranuclear inclusions are unstained. Nile blue. × 1,170

FIGURE 12.—Liver cells, 48 hours after infection. The fragments at the periphery of the nucleus are the only parts intensely stained for histone type of protein. Alkaline fast-green. × 1,170

FIGURE 13.—Liver cells, 42 hours after infection. Sickle-shaped bodies at the periphery of the nucleus show intense pyroninophilia that is abolished after RNase digestion. Methyl green-pyronin. × 1,170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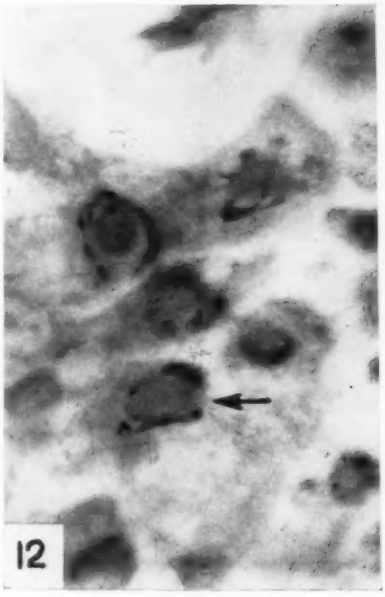
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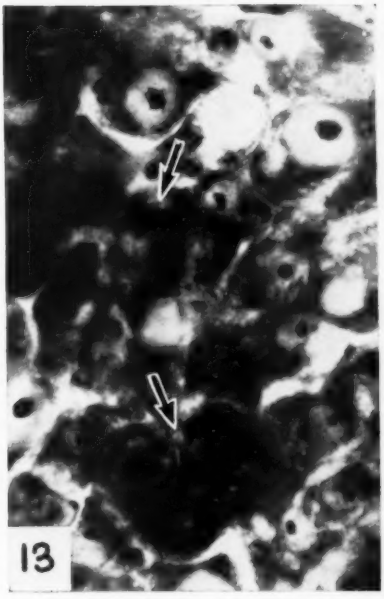
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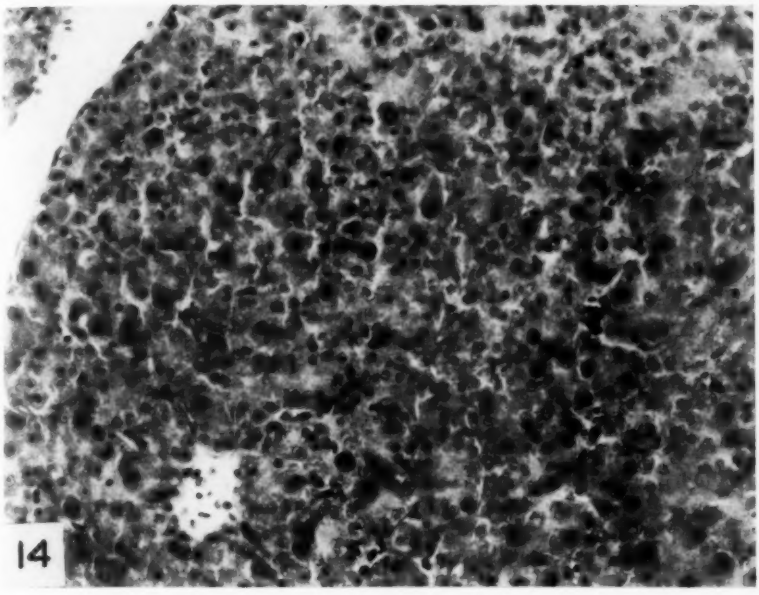
PLATE 90

Figures 14 to 28 are photographs of chicken embryo material.

FIGURE 14.—Liver of a 13-day-old embryo, 28 hours after inoculation of infectious material. Numerous cells show enlargement and increased density of the nucleus. Hematoxylin and eosin. × 360

FIGURE 15.—Same as figure 14, at higher magnification. *Note* vitreous appearance of the cytoplasm and numerous dense particles in the nucleus. Hematoxylin and eosin. × 1,080



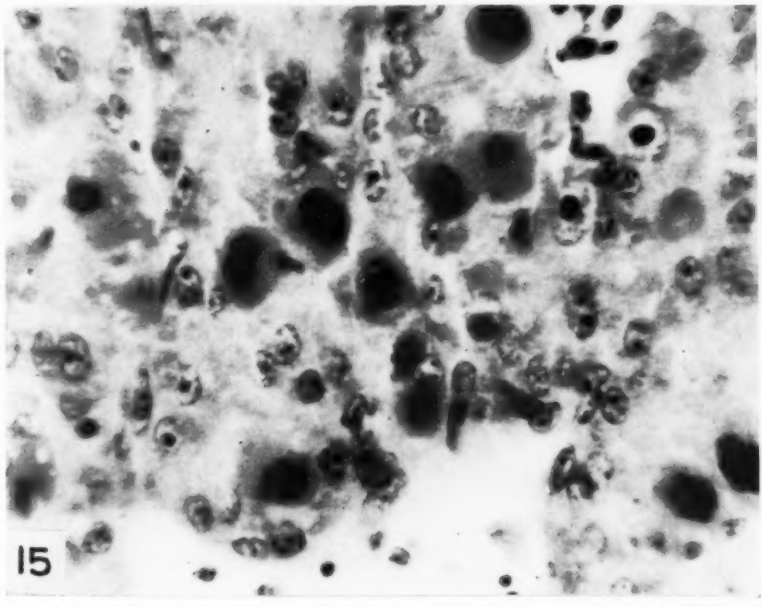


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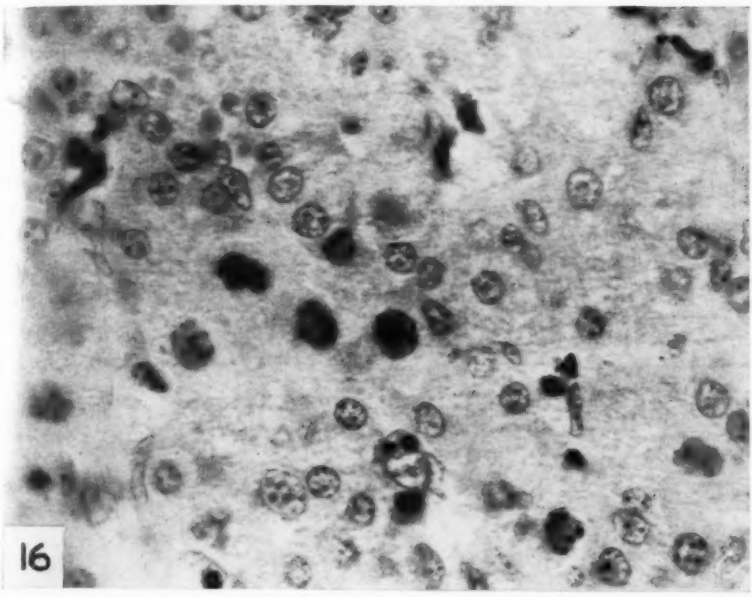
PLATE 91

FIGURE 16.—Same as figure 14. Four cells in the center of the field show intense staining for DNA in the nuclear sap and the small fragments. Nuclei of red blood cells are seen in the small sinusoids. Feulgen reaction. × 1,080

FIGURE 17.—Same as figure 14. Infected cells have numerous and swollen mitochondria. Iron hematoxylin. × 1,170

FIGURE 18.—Same as figure 14. *Arrows* indicate 2 cells with intranuclear inclusions. The small blocks are intensely positive while the rest of the nucleoplasm is lightly stained or negative. Tetrazonium reaction. × 1,170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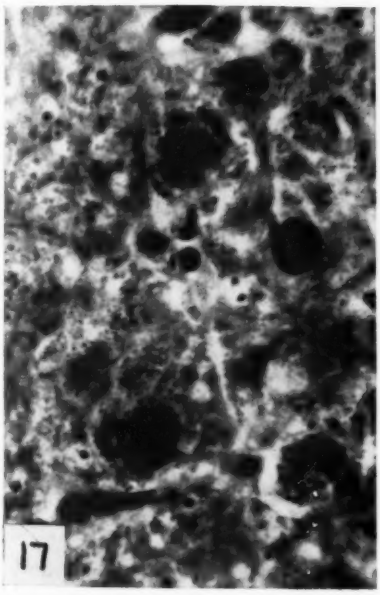
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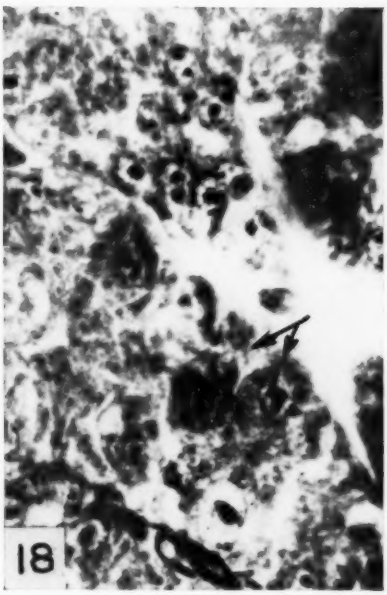
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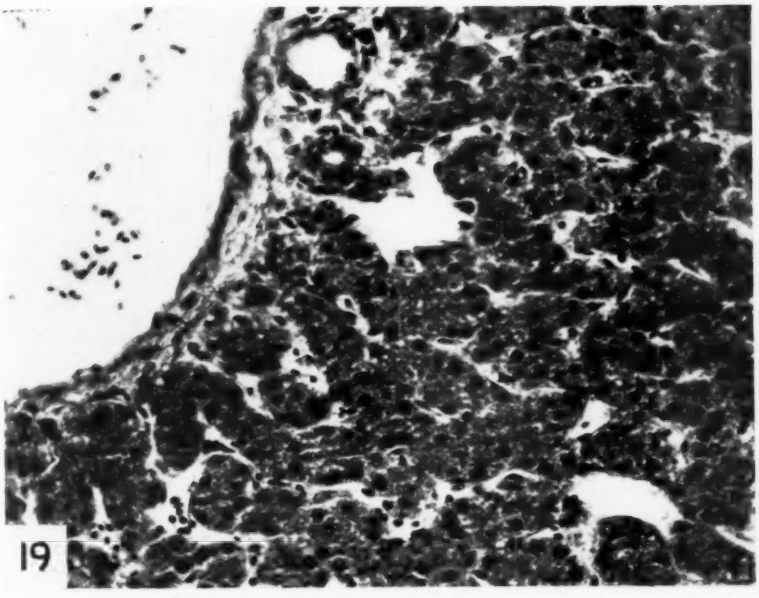
PLATE 92

FIGURE 19.—Liver of a 15-day-old embryo, 48 hours after inoculation with noninfectious material. Lumen of a portal vein is in the *upper-left* corner. A biliary duct is in the connective tissue of the portal space. Hematoxylin and eosin. × 400

FIGURE 20.—Liver of a 15-day-old embryo, 48 hours after inoculation with infectious material. *Note* some round and some polygonal cells in the loose mesenchyma of the portal area. Hematoxylin and eosin. × 400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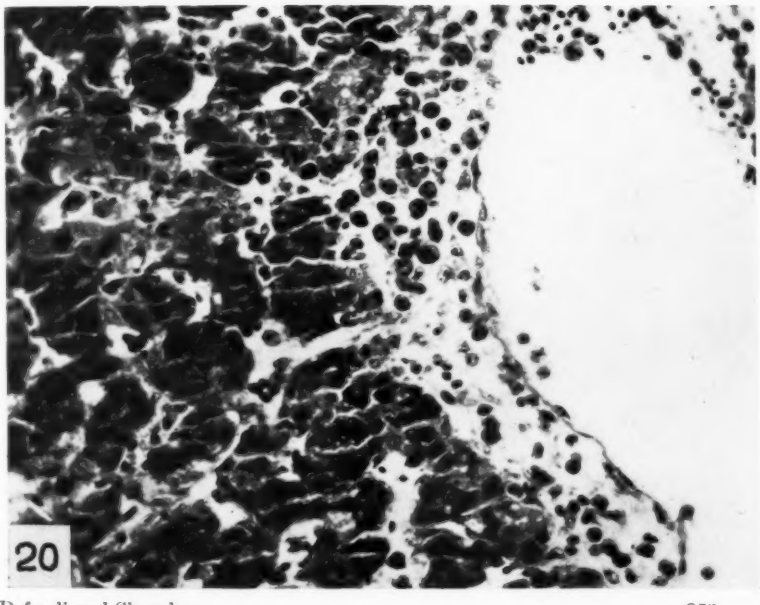
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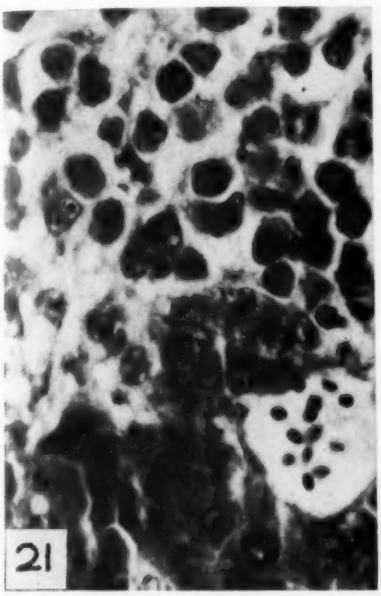
PLATE 93

FIGURE 21.—Same as figure 20, at higher magnification. Characteristics of the proliferating cells are evident; *note* the connection with the fine mesenchymal reticulum. Hematoxylin and eosin. × 800

FIGURE 22.—Myocardium of a 15-day-old embryo, 48 hours after inoculation with infectious material. In a perivascular space *note* large, round cells with vesicular nuclei and prominent nucleoli; intense basophilia of the cytoplasm. Hematoxylin and eosin. × 1,000

FIGURE 23.—Liver of a 16-day-old embryo, 72 hours after inoculation with infectious material. Thick, perivascular cuffing of the large, basophilic cells can be seen. *Note* identical cells protruding in the lumina of the sinusoids at *lower right*. Hematoxylin and eosin. × 400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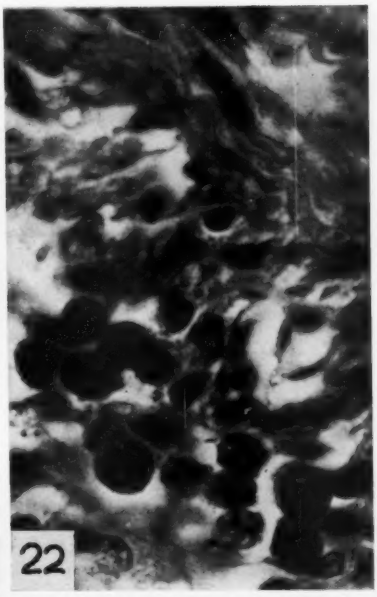
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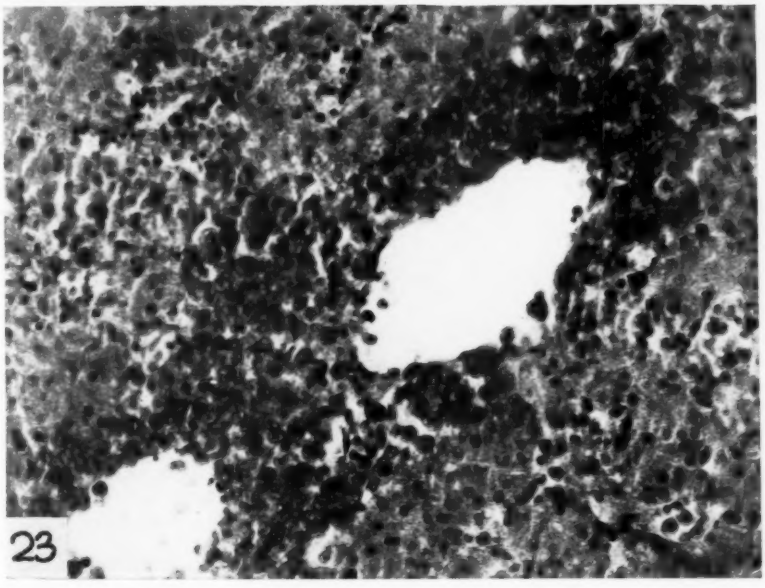
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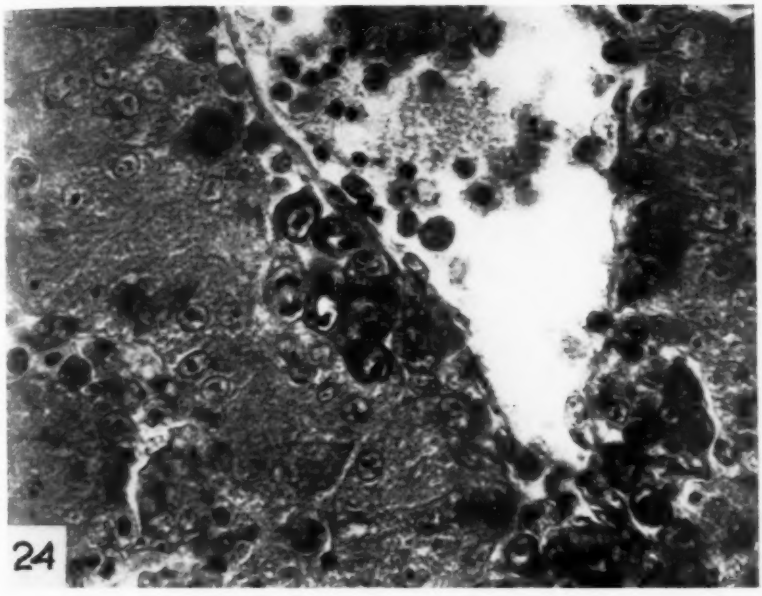
PLATE 94

FIGURE 24.—Same as figure 23, different field. *Note* large vesicular cells infiltrating the hepatic trabeculae; a cell in telophase in the center. Methylene blue and eosin. × 1,170

FIGURE 25.—Liver of a 15-day-old embryo, 50 hours after inoculation of infectious material. Large areas of hepatic necrosis are seen with hepatic cells still preserved around the portal spaces; *note* abnormal cells in some portal spaces and some nuclear inclusions at the terminal stage (round, very dark elements). Hematoxylin and eosin. × 180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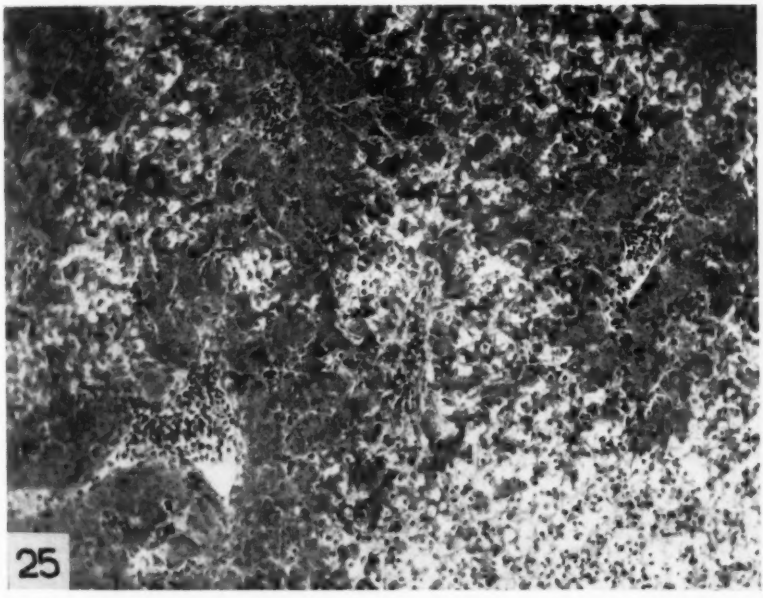
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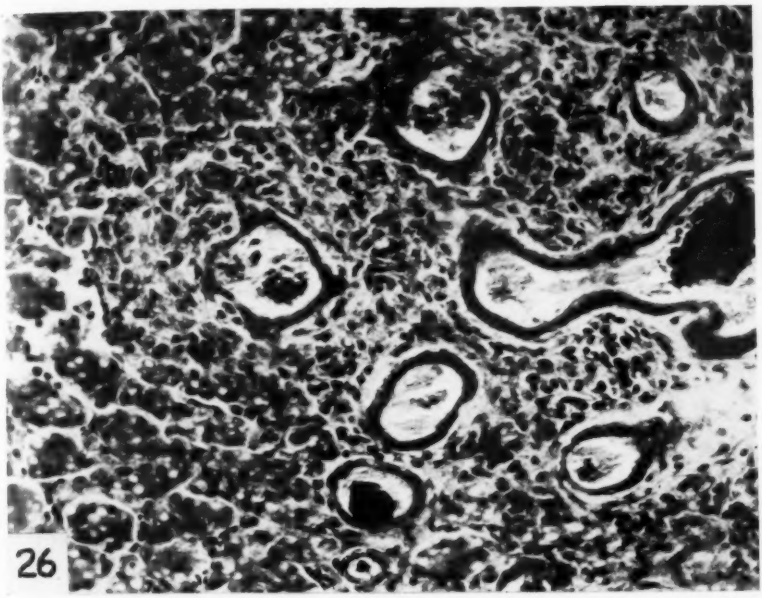
PLATE 95

FIGURE 26.—Liver of a 20-day-old embryo, on the 8th day after inoculation with infectious material. *Note* intense fibroblast and biliary-duct proliferation. The biliary ducts are dilated with accumulation of mucoid secretion and necrotic material in the lumen. Hematoxylin and eosin. × 360

FIGURE 27.—Spleen of a 14-day-old embryo, 48 hours after inoculation with non-infectious material. Multiform cell population with numerous myelocytes (darkly stained cells), some erythroblasts, and reticular cells can be seen. Hematoxylin and eosin. × 400

FIGURE 28.—Spleen of a 14-day-old embryo, 48 hours after inoculation with infectious material. *Note* uniformity of the cell population that consists of large reticular cells somewhat resembling the ones demonstrated in the liver and the myocardium. Hematoxylin and eosin. × 400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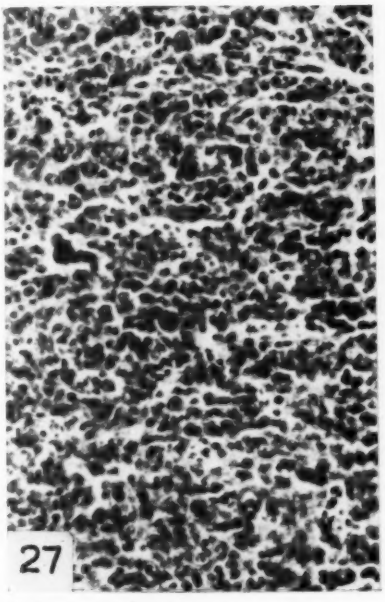
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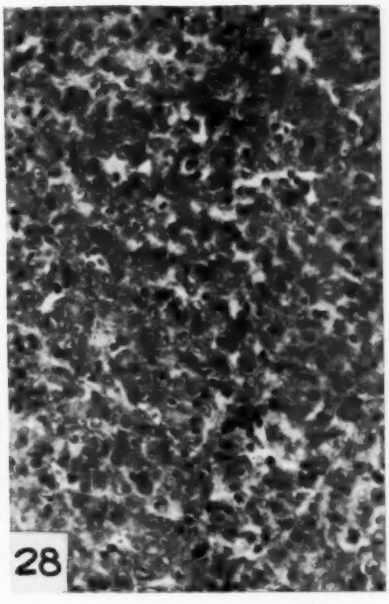

28

Measurement of Average and Total Cell Volume During the Growth of Ascites Tumors[1]

MARY E. RICE *and* EMMA SHELTON,[2] *Laboratory of Biology, National Cancer Institute,*[3] *Bethesda, Maryland*

Summary

The average volume of the cells of three lymphocytic ascites tumors was measured by two different methods: cell diameter and hematocrit-cell-count determinations. It was shown that the average cell volume decreased during the growth of two of the tumors. These changes in average cell volume during tumor growth could only be demonstrated by data obtained from cell-diameter measurements. It was found that, although the average cell volumes obtained by the microhematocrit-cell-count method were in over-all agreement with the cell-diameter measurements, the Poisson error in the cell count was large enough to mask the consistent decrease in cell volume that occurred during tumor growth. When the hematocrit method was used together with a dye-dilution method to determine the *total* volume of tumor cells in the ascitic fluid, a decrease in cell volume with increasing tumor age could be demonstrated. A plot of the increase in total cell number and total cell volume during tumor growth showed that in the later phases of growth the slope of the number curve exceeded that of the volume curve.—**J. Nat. Cancer Inst. 21: 961–971, 1958.**

The hematocrit, in combination with estimates of cell concentration, has been used extensively in measuring the average cell volume of ascites tumors (*1–5*). It appeared worthwhile, therefore, to compare the values obtained by this method with those obtained by the measurement of cell diameters. The latter method, although laborious, provided baseline values that were not complicated by errors caused by packing of cells or variations in enumerating the total number of cells.

Experimental Procedure

Three mouse ascites lymphomas were used in these studies: Lymphoid leukemia L1210, Lymphoma #3, and 2 lines of Lymphoma #1 designated as L#1-a and L#1-c. L#1-c was derived from a solid tumor at a later date

[1] Received for publication May 27, 1958.

[2] The authors wish to thank Mr. Nathan Mantel, Experimental Statistics Section, for his helpful advice in planning these experiments.

[3] National Institutes of Health, Public Health Service, U. S. Department of Health, Education, and Welfare.

than L#1-a, and the mean survival times of the tumors were, respectively, 14 to 16 days and 12 to 14 days. The origin of L1210 and L#1 has been described previously (*6*, *7*). L#3 originated in March, 1957, as a spontaneous lymphoma in a 5-month-old strain A/LN × A/HeN stock male mouse. The number of generations that these tumors have been carried in the ascites form is indicated in table 1.

The experiments were divided into 3 sets, the first 2 of which were concerned only with the estimation of average cell volume, while in the third, estimates of total cell volume were made in addition to average cell volume. The *average cell volume* was calculated by 2 methods: 1) from diameter measurements by the formula for the volume of a sphere, $V=\pi/6d^3$, and 2) by dividing the volume of cells per ml. derived from a hematocrit reading by the number of cells per ml. of ascitic fluid determined by a cell count. The *total volume* of cells was calculated from the percent volume of cells obtained from the hematocrit and the total volume of ascitic fluid as measured by a dye-dilution technique (*8*).

Experiment 1.—Average cell volumes were calculated from diameter measurements and compared with those calculated from the Van Allen hematocrit and cell count. Tumors L1210, L#3, and L#1-a were used at 3, 8, and 6 days, respectively, and the initial inoculum of tumor cells was not calculated. The average volume of the cells of L#1-c was calculated at 2-day intervals from the 6th to the 14th day of tumor growth after an initial inoculum of 6×10^6 cells.

Experiment 2.—The microhematocrit (*9*) as well as the Van Allen hematocrit (*10*) was used to measure average cell volume, and the volume obtained by each method was compared with that calculated from diameter measurements. L1210 was measured at 3 days of age, and L#3 at 8 days. The cells of L#1-c were measured at 8, 10, and 12 days after an initial inoculum of 6×10^6 cells.

Experiment 3.—The changes in cell volume during the growth of L#1-c and L1210 were studied. Mice were injected with 6×10^6 cells. The total number of cells and average and total volume of cells were determined at 24-hour intervals, beginning at 12 and 65 hours after inoculation and ending at 108 and 329 hours for L1210 and L#1-c, respectively.

Methods

Hematocrits.—Van Allen hematocrit tubes were centrifuged at 1600 × g for 1 hour at 0° to 5° C. in an International refrigerated centrifuge. The microhematocrit tubes were centrifuged at 30,000 × g for 5 minutes at room temperature.

Cell counts.—The method for estimating cell concentration has been described elsewhere (*11*). In brief, the ascitic fluid was diluted with a citric acid-crystal violet mixture and the cells counted in a hemacytometer. In the studies where cell size was related to the age of the tumor, the percent of pyknotic nuclei was also recorded.

Differential counts.—The percent of normal cells and tumor cells in the ascitic fluid was determined by a differential count of at least

1,000 cells from each sample of ascites. Details of the method have been previously described (*8*).

Cell diameter measurements.—Tumor cells were suspended in a hemacytometer and measured with a filar ocular micrometer in combination with a 40× oil-immersion objective. Measurements were recorded as micrometer units (1 unit = 0.183μ). Overlapping of the cells in the counting chamber was obviated by diluting the ascites with an appropriate amount of ascites serum. The cells tended to remain spherical and the few that departed from this shape were not measured. Macrophages were recognizable and were not measured, but lymphocytes and polymorphonuclear leukocytes (PMN) could not be distinguished from the tumor cells and a certain number were undoubtedly included in the measurement of the tumor cells. The error due to lymphocytes and PMN was minimized by using ascites in which the tumor cells constituted at least 80 percent of the total cell population. The percent of the lymphocytes and PMN in samples from all tumors varied from 0.3 to 16.7, with an average of 3.7.

Diameter measurements were also made on cells from the normal peritoneal fluid of 4 female strain A/LN × A/HeN mice 3 to 5 months of age. These measurements were used to calculate the normal cell volume (*see* later). Macrophages were measured separately, while lymphocytes and PMN were measured as one category.

Average cell volume.—The average tumor cell volume was calculated from the diameter measurements of 100 cells from each sample of ascites. In the first two experiments, for convenience, average cell volume was computed from data grouped into a frequency distribution. Measurements from each sample were segregated into classes with intervals of 3 to 5 micrometer units. The volume for the midpoint of each class was calculated and multiplied by the frequency of the interval, and the average volume for the sample was determined by summing the products and dividing by the number of measurements. In the third experiment, the volume of each cell was calculated and the sum of these was averaged to obtain an average volume.

The average volume of the normal cells was calculated by summing 300 individual volumes for each cell type and averaging the total. The average macrophage volume was 820 μ^3 (range, 387–2820) and the average PMN-lymphocyte volume was 248 μ^3 (range, 108–578).

Average cell volumes determined by the hematocrit method were obtained by dividing the volume of the cells per ml. by the number of cells per ml. of ascitic fluid.

Total cell number and total cell volume.—In the third experiment, total cell number and total cell volume were determined by a modification of the dye-dilution method of Révész and Klein (*5*) as described previously (*8*), except that the microhematocrit was used in the present experiments. In the calculation of total cell volume, cell counts were not required. The *total* cell volume was calculated by subtracting the serum volume from the total volume of ascites, but the estimation of the total *tumor* cell

volume necessitated a correction for the volume occupied by normal cells. It was possible to calculate the percent of the total cell volume attributable to normal cells by using percentages obtained from the differential counts and volumes obtained by the cell-diameter method.

$$\text{Percent total volume occupied by normal cells} = \frac{(vm)\ (nm) + (vl)\ (nl)}{(vt)\ (nt) + (vm)\ (nm) + (vl)\ (nl)}$$

where vm = average macrophage volume
vl = average lymphocyte + PMN volume
vt = average tumor cell volume
nm = percent macrophages
nl = percent lymphocytes + PMN
nt = percent tumor cells

The total normal cell volume was computed as the percent of the total volume occupied by normal cells times the total cell volume.

During the initial stages of tumor growth, when the tumor cells constituted less than 80 percent of the cell population, the average volume of the tumor cells was not measured but was assumed to remain constant. For L#1-c this period included the first 185 hours following inoculation and for L1210, the first 36 hours.

Results

The determinations of average cell volume are summarized in table 1. It is apparent from these data that centrifugation of the ascites in the Van Allen hematocrit at 1600 × g resulted in insufficient packing of the cells. The average cell volume determined by the Van Allen hematocrit was consistently greater than the volume calculated from the diameter measurements (ratio, 0.82). The microhematocrit method, on the other hand, with more complete packing gave an over-all average cell volume identical with that obtained by the diameter method (ratio, 1.01). When the individual ratios that make up this average are examined, however, it is obvious that there is considerable internal variation. The variability is probably a reflection of the Poisson error inherent in the cell-count procedure, which in these experiments was estimated to be 7 to 10 percent. The determination of average cell volume by the hematocrit-cell-count method can thus be only as accurate as the estimate of cell number. It should be noted that there was no apparent correlation between the number of dead cells and the variation in the hematocrit measurements. The percent of dead cells in L#1-c remained relatively low throughout the growth of the tumor, exceeding 2 percent in only 2 instances in experiment 1, namely, 5 percent at 12 days and 9 percent at 14 days. In L1210 (expt. 3), the percent of dead cells was never greater than 1.

The individual measurements of average cell volume obtained by the hematocrit-cell-count method on tumor L#1-c and L1210 (expt. 3) varied at random with no relation to the age of the tumor (table 1). In contrast, the diameter method demonstrated a decrease in the average volume of the cells as the tumors increased in age. This decrease in cell volume was

TABLE 1.—*Summary of average volume measurements*

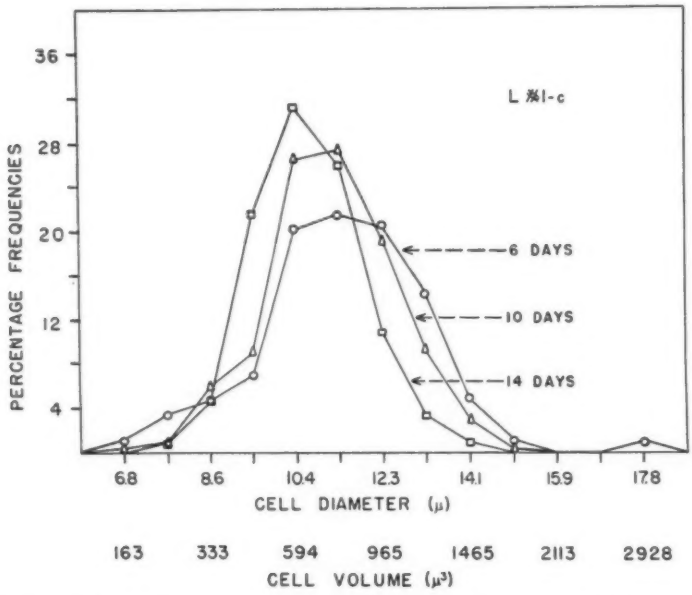
Expt. No.	Tumor	Generation No.	Percent tumor cells	Number of samples	Age of tumor (*days*)	Average cell volume by diameter method (μ^3)	Average cell volume by Van Allen hematocrit (μ^3)	Ratio: volume by diameter / volume by Van Allen hematocrit	Average cell volume by microhematocrit (μ^3)	Ratio: volume by diameter / volume by microhematocrit
1	Lymphoma #3	21	—	5(6)*	8	369	448	0. 82		
	Lymphoid leukemia L1210	160–186	93(86–97)†	10	3	748	974	0. 77		
	Lymphoma #1-a	77–92	93(89–96)	10(19)	6	659	853	0. 77		
	Lymphoma #1-c	7, 8	83, 80	2(4)	6	832	854	0. 97		
		7	92, 94	2	8	774	879	0. 88		
		7	92, 96	2	10	753	827	0. 91		
		7	97, 98	2	12	667	798	0. 84		
		7	99, 98	2	14	652	946	0. 69		
2	Lymphoma #3	27	—	5	8	410	436	0. 94	382	1. 07
	Lymphoid leukemia L1210	201	91(87–93)	5	3	867	1068	0. 81	939	0. 92
	Lymphoma #1-c	11	89, 94	2	8	831	904	0. 92	791	1. 05
		11	97, 95	2	10	732	935	0. 78	837	0. 87
		11	98, 98	2	12	717	944	0. 76	831	0. 86
3	Lymphoma #1-c	15	91(89–95)	3	8	830			726	1. 14
		15	96(95–98)	3	9	798			678	1. 18
		15	98(96–98)	3	11	765			742	1. 03
		15	96, 96	2	12	742			789	0. 94
		15	97, 99	2	13	707			736	0. 96
		15	98(98–99)	3	14	699			678	0. 97
	Lymphoid leukemia L1210	247	90(89–90)	3	2	1051			928	1. 13
		247	91(90–93)	3	3	959			1032	0. 93
		247	94(93–94)	3	4	893			912	0. 95
		247	95(95–96)	3	5	767			846	0. 87
	Average ratio							0. 82‡		1. 01‡

*Where number of samples does not equal number of animals, figures in parentheses give the total number of animals from which ascites was pooled to obtain samples.
†Range.
‡Average of total individual samples.

observed in more than one tumor generation (table 2). The absolute decrease and rate of decrease in cell size is greater for L1210 than for L#1-c. This is, perhaps, a reflection of the speed with which the two tumors kill the host and the resulting cachexia of the host in the terminal stages of the disease. L1210 kills the host in half the time of L#1-c.

As a measure of cell volume, the hematocrit furnishes only one piece of information, namely, the percent of the total volume of the ascites that is occupied by cells. If the number of cells per ml. of ascites is known, it is possible to obtain an average value for the volume of a cell in the ascites. Diameter measurements of individual cells, on the other hand, provide data that can be arranged to furnish additional information about the volume changes that take place in the cells during the growth of the tumor. Frequency distributions of the cell-diameter measurements of L#1-c and L1210 show that the decrease in cell volume during tumor growth is due to a shift in the mode toward a smaller cell size, with a concomitant decrease in the number of large cells and an increase in the number of small cells (text-figs. 1 and 2).

In experiment 3 the total number and total volume of tumor cells was measured during the growth of L#1-c and L1210. The values for the volume of tumor cells were obtained by subtracting the normal cell volume from the total cell volume. It was found that the total volume of normal



TEXT-FIGURE 1.—Frequency polygons plotted from cell-diameter measurements of L#1-c (expt. 1). Each curve represents a total of 200 cells. Eight animals were used, 4 for the 6-day measurement and 2 each for the 10- and 14-day measurements. Abscissa is scaled in ocular micrometer units which are converted to diameter and volume values.

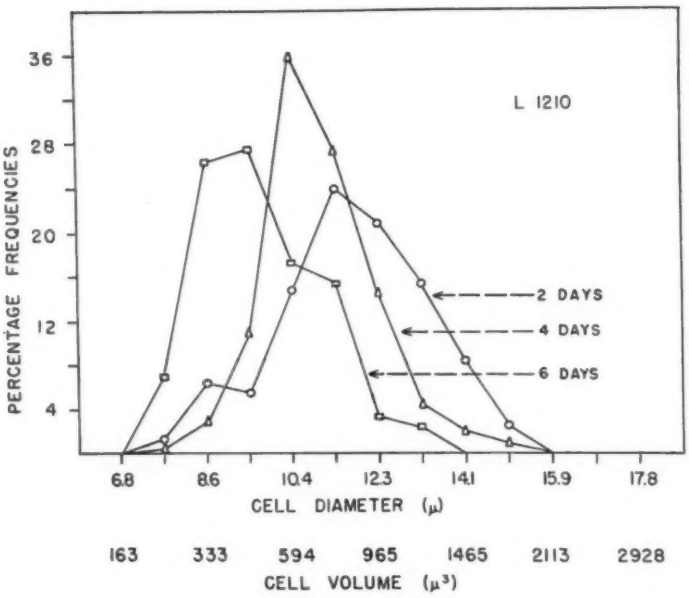
TABLE 2.—*Decrease in average cell volume with time**

Lymphoid leukemia L1210							Lymphoma #1-c								
Expt. No.	Generation No.	Days					Expt. No.	Generation No.	Days						
		2	3	4	5	6			6	8	9	10	11	12	13	14
–†	208	882	–	765	–	529	1	7	815	771	–	758	–	696	–	657
		920	–	717	–	525			848‡	776	–	748	–	639	–	648
3	247	1138	993	900	764	–	2	11	–	817	–	721	–	729	–	714
		1004	960	922	766	–			–	844	–	743	–	707	–	682
		1012	923	858	770	–	3	15	–	844	790	–	803	698	676	707
									–	818	861	–	763	785	738	679
									–	828	744	–	725	–	–	711
Average		991	959	832	767	527	Average		832	814	798	743	764	709	707	685

*Numbers in table are averages of diamater measurements of 100 cells expressed in μ^3. A single mouse was used for each measurement except for L#1-c at 6 days when the ascites from 2 mice was pooled.

†Separate series of diameter measurements only.

‡Generation 8.



TEXT-FIGURE 2.—Frequency polygons plotted from cell-diameter measurements of L1210. Each curve represents a total of 200 cells from 2 animals of tumor generation 208. Abscissa is scaled in ocular micrometer units which are converted to diameter and volume values.

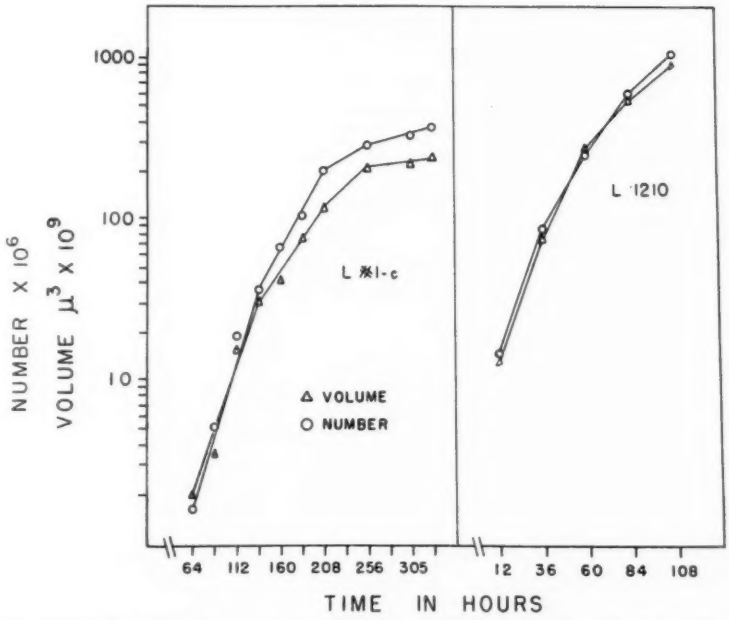
cells of L#1-c remained constant at $4 \times 10^9\ \mu^3$ throughout the growth of the tumor. The total volume of normal cells associated with L1210, however, increased from $5 \times 10^9\ \mu^3$ to $35 \times 10^9\ \mu^3$ during the period of measurement.

During the first stages of tumor growth the slopes of volume and number (text-fig. 3) closely approximate each other in both tumors, but in L#1-c, there is marked divergence in the later stages of growth. In L1210 the slight divergence of these slopes is more significant than it appears because the tumor becomes bloody at 84 hours and, despite the added volume of the erythrocytes, the total volume of cells decreases relative to number. The observed divergence of the curves for volume and number of cells suggests a confirmation of the decrease in average cell volume demonstrated by cell-diameter measurements.

Discussion

Lucké and Berwick (*4*) used the microhematocrit in conjunction with cell counts to determine the average size of Ehrlich ascites tumor cells and found good agreement with cell-diameter measurements. This is confirmed by our results which show an average ratio of 1.0 between the two methods. It is obvious that $1600 \times g$, the maximum force at which it was possible to centrifuge the Van Allen hematocrit, was not sufficient

for complete packing of the cells. The 18 percent difference between the volume measured by the diameter method and the Van Allen hematocrit method was eliminated by using the microhematocrit at a force of 30,000 × g. The fact that an average ratio of 1.0 was obtained between diameter and microhematocrit methods indicates that errors due to the presence of normal cells or to variation in cell count are negligible when large numbers of measurements are made. However, the error in cell counting inherent in the hematocrit-cell-count method was sufficiently large to mask the demonstration of a decrease in average cell size during tumor growth.



TEXT-FIGURE 3.—Growth curves of L#1-c and L1210 comparing number and volume of tumor cells. The points are averages of values from 3 mice, except the penultimate of L#1-c, which are averages of 2 mice.

Klein and Révész (*2*) and Lucké and Berwick (*4*), using the hematocrit technique, found no change in the average cell volume during the growth of the Ehrlich ascites tumor from 3 to 16 days, but Levy *et al.* (*3*) reported a striking decrease in the average volume of DBA lymphoma cells during the growth of this tumor. The reality of the decrease in cell size of the DBA lymphoma is not completely certain. In their quantitative studies of the DBA, EL4, and 6C3HED lymphomas, Révész and Klein (*5*) were able to show that the pronounced decrease in cell volume, which occurred with increasing age of these tumors, was strongly correlated with an increase in the number of dead cells. A change in the size of the living tumor cells, therefore, was not clearly demonstrated. Révész and Klein in-

dicated, however, that some of their data suggested a real decrease in the volume of the viable cells.

The present work shows definitely that a decrease in average tumor-cell volume accompanies increasing tumor age. This decrease in cell volume is probably associated with the accumulation of metabolic wastes in the ascitic fluid, the anoxia, and the limitation of the nutrient supply that develops as the number of tumor cells increases in the peritoneal cavity. It is of interest in this connection that Waymouth (*12*) noted a decrease in the average size of strain L cells growing in crowded conditions in tissue culture.

When the relatively small cells from well-advanced ascites tumors are inoculated into a new host, a rather rapid increase in average cell volume must occur. For example, in the case of L1210, if cells of a 6-day-old tumor with an average volume of 527 μ^3 are injected into a new host, an average increase of 464 μ^3 would be accomplished in 2 days after tumor inoculation (table 2). Lucké and Berwick, in their work with the Ehrlich ascites tumor, suggest that during the first 2 days of growth the cells of the tumors are "slightly but significantly larger than during the remainder of the growth cycle," and such an initial size increase is also implied in the studies of Klein and Révész.

The hematocrit, if used to estimate *total* tumor-cell volume, is a simple technique for the measurement of growth of ascites tumor cells, which does not involve cell counting. The speed of centrifugation and the amount of cell packing are not important as long as they stay constant, and, provided the error contributed by normal cells or other extraneous material can be accounted for during the early stages of growth, the slope of the volume increase will approximate that of tumor cell number. But if growth in terms of total volume is to be compared with, or substituted for, growth in terms of total numbers of cells, errors due to variations in the volume of the individual cells should be considered. If it is desirable to determine *average* cell volume by the hematocrit-cell-count method, many samples should be used and the rate and duration of centrifugation should be carefully controlled.

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Concentrative Uptake of Azaserine by Neoplastic Plasma Cells and Lymphocytes [1,2]

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Summary

Five ascites tumor strains were investigated for their ability to concentrate azaserine intracellularly *in vitro*. The azaserine-sensitive 70429/S plasma-cell tumor accumulated azaserine to intracellular concentrations as high as 30 times the external concentration. The 2 azaserine-resistant sublines, 70429/AZ-R-1 and 70429/AZ-R-5, concentrated the drug less efficiently than the sensitive parent strain at extracellular concentrations below 10 μg. per ml. At higher concentrations there was no correlation between drug sensitivity and drug uptake. The spontaneously resistant 6C3HED lymphosarcoma accumulated azaserine least efficiently: At all concentrations tested, azaserine uptake was less than that of the plasma-cell tumors. The rate and extent of azaserine uptake was inhibited by 2,4-dinitrophenol, sodium azide, and *p*-chloromercuribenzoic acid, which also reversed previous drug uptake by 50 percent or more. Many amino acids inhibit azaserine accumulation and displace it from its intracellular site; both D- and L-isomers are active. A free α-amino and carboxyl group appear essential for blocking activity.—**J. Nat. Cancer Inst. 21: 973–984, 1958.**

Azaserine (O-diazoacetyl-L-serine) has been of experimental interest as an antitumor agent and antibiotic. It inhibits purine synthesis in both bacterial (*1*) and avian systems (*2*) and has also been shown to prevent the utilization of free intracellular amino acids in yeast (*3*).

This investigation examines the mode and extent of uptake of the drug in 5 ascites tumor systems. The azaserine-sensitive 70429/S plasma-cell tumor was compared with its 2 resistant sublines, 70429/AZ-R-1 and 70429/AZ-R-5. Further comparisons were made with the Gardner 6C3HED lymphosarcoma and Dalton thymoma (DBA/2), which are naturally resistant to the drug.

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[3] The author is grateful to Dr. T. S. Hauschka for his constant interest in and support of these studies. He also furnished mice carrying the 6C3HED and DBA/2 tumors as well as stock C3H and DBA/2 mice. Dr. Michael Potter supplied the plasma cell tumors 70429/S, 70429/AZ-R-1, and 70429/AZ-R-5, and gave advice on their propagation. He also donated a supply of D-azaserine. Dr. Robert Guthrie provided laboratory facilities.

When these studies were concluded, the investigation by Jacquez (*4*) on azaserine uptake by Ehrlich ascites cells appeared, and it is discussed in the light of the present findings.

Materials and Methods

Origin and maintenance of tumors.—Five ascites tumors were grown without azaserine. The Gardner lymphosarcoma 6C3HED and the Dalton thymoma (DBA/2) have been described previously by Klein (*5*) and Hauschka (*6*). The tumors were transferred every 8 and 10 days into strain C3H/St and DBA/2 mice respectively, by intraperitoneal injections of 0.2 ml. undiluted ascites (about 50 to 80 million cells). To determine the azaserine sensitivity of 6C3HED and DBA/2 tumors, mice were treated during 43 and 35 successive tumor transfers respectively, with 5.5 mg. per kg. azaserine intraperitoneally 5 times a week. Survival times did not differ significantly from those of untreated mice. The azaserine-sensitive 70429/S plasma-cell neoplasm and its resistant derivatives, 70429/AZ-R-1 and 70429/AZ-R-5, described by Potter *et al.* (*7*), were maintained by routine transplantation of 0.2 ml. ascites every 8 to 14 days into 2- to 3-month-old C3H/He mice.

Determination of azaserine[4].—A microbiological assay procedure was developed with *Escherichia coli* B/r as the test organism. Incubations were carried out at 37° C. Growth from a 12- to 15-hour nutrient agar slant was suspended in mammalian Ringer's solution. It was mixed in a medium that contained 7.1 gm. K_2HPO_4, 3 gm. KH_2PO_4, 1 gm. $(NH_4)_2SO_4$, 10 mg. $MgSO_4 \cdot 7H_2O$, 0.5 mg. $FeSO_4 \cdot 7H_2O$, 10 mg. $CaCl_2$ per liter of distilled water, and 1.5 percent agar. A 0.4 percent solution of glycerol was autoclaved separately and the medium adjusted to *p*H 7.0. This mixture was poured to a ½-inch depth in a large pyrex baking dish over an equal amount of the same medium, previously solidified and dried overnight. Refrigeration of these pour plates for about 6 hours improved the assay by sharpening the delineation of the inhibition zones.

Solutions for assay were added in 0.2 ml. amounts, or less, on Schleicher and Schuell #740-E assay discs spaced on the agar. The diameters of the inhibition zones were read after 12 to 20 hours' incubation at 37° C.

Standard azaserine values usually ranging from 1.3 to 20 μg. gave linear curves, when plotted semilogarithmically against the zone diameters, corresponding respectively to 10 to 25 mm. after subtraction of the disc diameters.

This assay is as sensitive as the spectrophotometric procedure of Jacquez (*4*). Although it is less sensitive than the *Kloeckera brevis* assay of Kohberger *et al.* (*8*), it has the advantage of being unaffected by the amount of amino acids and cell extracts in the experiments. The inhibitors 2,4-dinitrophenol, *p*-chloromercuribenzoic acid, sodium azide, Versene, α,α'-dipyridyl, 8-hydroxyquinoline and sodium malonate did not affect the azaserine zones in the relative concentrations used.

[4] Parke, Davis & Company, Detroit, Mich., and the Cancer Chemotherapy National Service Center, National Institutes of Health, Bethesda, Md., provided gifts of azaserine.

Collection and preparation of ascites cells.—Mice were killed by cervical dislocation and the ascites removed from the abdominal cavity with a syringe. All subsequent manipulations were carried out at 0° to 4° C., unless otherwise specified. After centrifuging and decanting the supernatant plasma, the cells were washed and resuspended in mammalian Ringer's solution containing 0.0125 M Na_2HPO_4, *p*H 7.0. The total volume of the final cell suspension was never more than that of the original. Cells not used immediately were refrigerated. They were always used on the same day and usually within an hour after harvesting. No detectable difference in azaserine uptake was noted between nonbloody and bloody ascites preparations.

Exposure of cells to azaserine and method of assay.—In a typical experiment, suspensions containing 0.02 to 0.05 ml. packed cells in a final volume of 1.5 ml. were exposed to treatment in small test tubes at 37° C. for 20 to 30 minutes and shaken to obtain an even suspension. Further incubation did not increase azaserine uptake. The supernatant containing all extracellular azaserine was then separated by centrifugation and assayed. The packed cells were drained for several minutes and the inside of the tubes wiped dry without disturbing the pellets. The cells were resuspended in 0.3 ml. distilled water and disrupted by heating to 60° C. for 1 minute without destroying azaserine significantly. After cooling, the cell debris was spun down at high speed and the supernatant, which contained all the intracellular azaserine, was assayed. This method produced total lysis provided the packed-cell volume per tube was not more than 0.1 ml. Replicates without azaserine were used in each experiment. The packed-cell-volume determinations were made in a hematocrit tube. Replicates without cells were assayed immediately for determination of initial extracellular azaserine. All values were determined in duplicate.

The cells appeared to maintain their azaserine levels during manipulation, since 3 consecutive washings in mammalian Ringer's solution removed only about 35 percent of the azaserine content of unwashed cells. There appeared to be little destruction of azaserine by the tumor cells throughout the experiments. Even at the lowest levels the azaserine recovered from the cells and supernatant approximated that from control incubations of azaserine solutions.

Calculations.—The extracellular azaserine concentration (C_e) was calculated in μg. azaserine per ml. of extracellular fluid and the intracellular azaserine concentration (C_i) in μg. azaserine per cell ml. as:

$$\left(10\, w_i \frac{v_i}{v_p} - s\ 10\, w_e\right)/(1-s).$$

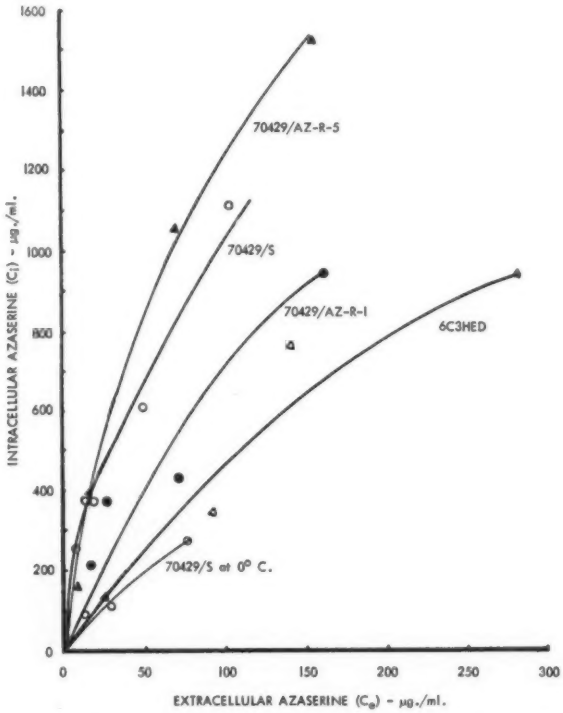
The symbol w represents the μg. azaserine assayed in 0.1 ml. of the sample; w_e, the extracellular azaserine in the supernatant before lysis; and w_i, the pellet azaserine assayed from the lysate. The symbol v refers to volume in ml.; v_p, the pellet volume before lysis; and v_i, the total volume including cell debris after lysis. The symbol s represents the extracellular water

space, which was calculated from a series of separate experiments as 0.33 of the pellet volume. This value was the average ratio of azaserine content of the pellet : supernatant when the cells were centrifuged at 0° C. immediately on addition of azaserine. Although this procedure may not unquestionably exclude intracellular entry, it would not give serious errors in final calculations.

Results

Concentrative Uptake of Azaserine

Azaserine is concentrated by azaserine-sensitive as well as azaserine-resistant ascites cells, as demonstrated in text-figure 1. The intracellular concentration is dependent over a large range on the extracellular concentration in the steady state. The curves resemble the initial segment

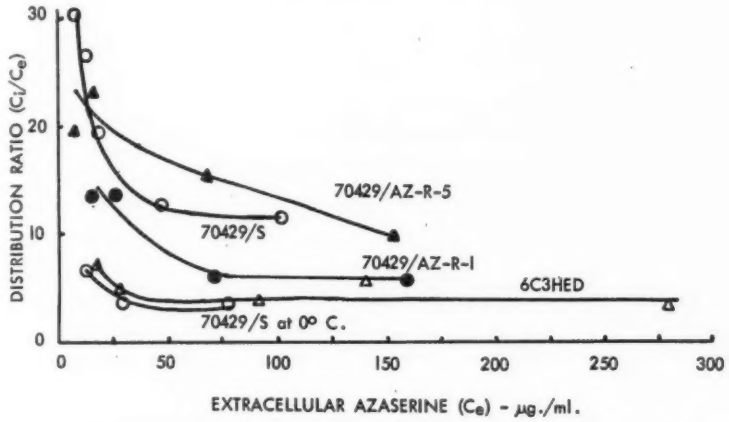


TEXT-FIGURE 1.—Intracellular accumulation of azaserine in 4 ascites tumors at different extracellular concentrations.

Strain 6C3HED, generation 306, at 11 days after inoculation.
Strain 70429/S, generation 62, at 11 days after inoculation.
Strain 70429/AZ-R-1, generation 50, at 21 days after inoculation.
Strain 70429/AZ-R-5, generation 51, at 15 days after inoculation.

All cells were incubated with azaserine for 30 minutes at 37° C., except when otherwise noted.

of the curve obtained with Ehrlich ascites cells (*4*). The slopes of the curves are variable, the 6C3HED lymphosarcoma producing the most gradual and the 70429/AZ-R-5 plasma-cell tumor the steepest slope. The slopes produced by plasma-cell tumors 70429/S and 70429/AZ-R-1 fall in between. The extent of azaserine accumulation of the DBA/2 thymoma is of the same order of magnitude as that of the 6C3HED strain. Although plasma-cell tumors 70429/AZ-R-1 and AZ-R-5 and the 6C3HED lymphosarcoma are azaserine-resistant, they nevertheless concentrate azaserine extensively *in vitro*. The efficiency of this concentration is elucidated in text-figure 2, where the distribution ratio in the steady state is plotted against the final external drug concentration. The sensitive 70429/S plasma-cell tumor was consistently the most efficient and the 6C3HED lymphosarcoma the least efficient in concentrating azaserine at low extracellular levels.



TEXT-FIGURE 2.—Distribution ratios of azaserine in 4 ascites tumors at different extracellular concentrations. Data are derived from text-figure 1.

At lower drug concentrations which would be closer to therapeutic levels, the concentrating efficiencies of the different tumors increased but not consistently. Extrapolating down to selectively toxic levels, we found that the sensitive parent became more efficient than either of its resistant derivatives, even though the difference was not apparent at higher drug concentrations. Thus, variation in efficiency of concentrative uptake does correlate with therapeutic resistance and this step may determine differences in drug susceptibility of the 3 tumors. Consistently, the 6C3HED lymphosarcoma, which is naturally resistant to azaserine, concentrated the drug the least.

Although tumor 70429/S concentrated azaserine at 0° C., the magnitude of the uptake was about one fourth of that at 37° C., as seen in text-figures 1 and 2. More prolonged incubation at 0° C. did not improve uptake; hence, the decrease is due to a lowered extent rather than a lowered rate

of uptake. This finding differs from that of Jacquez (*4*) who found no uptake at 0° C. with Ehrlich ascites tumors. If the azaserine uptake were due to adsorption rather than to an enzyme-mediated uptake, one would expect a greater drug uptake at the lower temperature, which is not the case.

Inhibition of Uptake

Table 1 shows the effect of certain inhibitors on azaserine uptake by strains 6C3HED, 70429/S, and 70429/AZ-R-5. Sodium azide, *p*-chloromercuribenzoic acid, and 2,4-dinitrophenol inhibited simultaneous uptake and reversed prior uptake 50 percent or more, whereas metal chelating agents, such as 10^{-3}M 8-hydroxyquinoline, Versene, or α,α'-dipyridyl, showed 10 percent inhibition or less. Sodium malonate at a concentration of 10^{-3}M was without effect.

In a time experiment it was found that on addition of azaserine to cells of strain 70429/AZ-R-5, which had been preincubated for 30 minutes with 2,4-dinitrophenol or *p*-chloromercuribenzoate, the rate as well as the extent of drug uptake was depressed and reached equilibrium after 20 minutes, just as the controls without inhibition.

The inhibitory effects of dinitrophenol and sodium azide suggest linkage of the concentrating system to energy-yielding metabolic reactions of the cell. On the other hand, the inhibitory action of *p*-chloromercuribenzoic acid, a mercaptide-forming agent, might be due to random damage to cellular structures. Studies by Shacter (*9*) on changes in inorganic phosphate levels of Ehrlich ascites cells inhibited with *p*-chloromercuribenzoate substantiate this point of view.

Effect of Amino Acids and Related Compounds on Azaserine Uptake

Numerous studies on azaserine have implicated the drug as an inhibitor of several biological reactions. In every case the inhibition could be prevented or reversed by one or more amino acids, which differed for each system. Thus, the synthesis of formylglycinamidine ribotide from its amide in pigeon-liver extracts is relieved but not reversed by glutamine (*2*); the synthesis of 5-amino-4-imidazolecarboxamide in a purine-requiring strain of *E. coli* is reversed by the aromatic amino acids (*1*); induced maltase synthesis in *Saccharomyces cerevisiae* is reversed fully by leucine and partially by phenylalanine, isoleucine, and many other amino acids (*3*).

It was of interest to find out whether amino acids would also antagonize azaserine uptake when ascites strains 6C3HED and 70429/S were used. The following amino acids were found to inhibit drug uptake at least 75 percent at an azaserine : L-amino acid ratio of 1 : 20: L-alanine, L-valine, L-norvaline, DL-isoleucine, DL-norleucine, L-serine, L-threonine, L-cysteine hydrochloride, L-glutamine, L-ethionine, and L-methionine. Among the aromatic amino acids, tyrosine was the most effective, and phenylalanine the least effective, in blocking azaserine uptake, producing 69 and 33 percent inhibition, respectively.

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TABLE 1.—*Effect of inhibitors on intracellular azaserine uptake of different ascites tumors*

Expt. No.	Ascites strain	Extracellular azaserine (C_e, μg./ml.)	Distribution ratio ($\frac{C_i}{C_e}$)	Inhibitors								Experimental conditions
				p-Chloromercuribenzoic acid		Sodium azide		2,4-Dinitrophenol		8-Hydroxyquinoline		
				M	Percent of control	M	Percent of control	M	Percent of control	M	Percent of control	
1	6C3HED*	110	7.0	2×10^{-4}	26	10^{-2}	35	1×10^{-3}	35			Preincubated with inhibitors for 30 min. and additional incubation with azaserine for 20 min.
					32		58		58			Preincubated with azaserine for 20 min. and additional incubation with inhibitors for 30 min.
2	70429/S†	140	6.0	10^{-3}	21	10^{-2}	32			10^{-3}	89	Preincubated with inhibitors for 30 min. and additional incubation with azaserine for 30 min.
3	70429/AZ-R-5‡	120	7.0	2×10^{-4}	21			5×10^{-4}	32			Preincubated with inhibitors for 30 min. and additional incubation with azaserine for 30 min.

*Generation 32, harvested at 11 days.
†Generation 59, harvested at 10 days.
‡Generation 50, harvested at 16 days.

In strain 6C3HED, D-azaserine inhibited the uptake of the L-isomer by 74 percent at a molar ratio of 10 : 1.

Several amino acids were tested for their ability to displace the drug from its intracellular site. In such an experiment a large volume of cells was exposed to azaserine for 30 minutes at 37° C. and, after cooling to 0° C., was divided into small aliquots. These were first washed to remove all azaserine and then aerated for 5 minutes at 37° C. with different amino acids. They were then assayed for azaserine.

In an experiment in which the initial concentration of azaserine to L-amino acid was 1 : 3 mg. per ml., L-glutamine and L-methionine displaced between 90 and 100 percent of azaserine from the cells, compared to the mammalian Ringer-washed and aerated control. L-glycine, L-alanine, and L-valine displaced between 80 and 90 percent; L-serine, L-proline, L-asparagine, L-isoleucine, L-aspartic acid, L-threonine, and L-leucine displaced between 50 and 80 percent; L-glutamic acid, L-arginine, and L-lysine displaced less than 50 percent. Phenylalanine was the only amino acid that was inactive when tested. When the D-isomers of methionine, alanine, and valine were tested for displacement activity, with strain DBA/2, D-methionine was found to be slightly more active than L-methionine, whereas D-alanine and D-valine were one third and one seventh as active as L-alanine and L-valine, respectively.

In an effort to elucidate the functional groups required for blocking azaserine uptake, compounds structurally related to active amino acids were also tested. As demonstrated in table 2, absence of the amino group, as exemplified by sodium lactate, propionate, and α-ketoglutarate, and loss of the carboxyl group, as exemplified by ethylamine, led to partial or total loss of blocking activity. A secondary amino acid was less active than the primary one. Moving the amino group from an α to a β position as shown by the loss of activity from β-alanine and β-aminoisobutyrate indicated the necessity for an α-amino group. In another experiment, the acyl amino acids *N*-acetylglycine and DL-*N*-acetylmethionine were found to be almost ineffective as blocking agents; in their presence azaserine uptake was 95 percent of that of the control.

Among the amino acids effectively inhibiting intracellular azaserine uptake in whole cells, glutamine holds a special position because it blocks azaserine inhibition of purine synthesis by cell-free extracts. Since this inhibition is competitive, it was of interest to compare it with the type of antagonism found in our system.

As may be seen in table 3, the glutamine-induced reversal of azaserine uptake is dependent on the relative concentrations of both compounds over a narrow range with strain 70429/S. In strains 70429/AZ-R-5 and DBA/2, the inhibition of azaserine uptake is increased by an increase in glutamine concentration alone.

These inhibition studies demonstrate the heterogeneous response of the 3 strains. It is interesting to note, for instance, that 25 μmoles of glutamine reduced the distribution ratio of strain 70429/S to 27 percent

TABLE 2.—*Effect of amino acids and structurally related compounds on azaserine uptake*

Expt. No.	Ascites strain	Extracellular azaserine (C_e, μg./ml.)	Distribution ratio $\left(\frac{C_i}{C_e}\right)$	Compounds added*	Percent of control
1	6C3HED†	230	4.7	None	100
				α-Alanine	<6
				β-Alanine	78
				Sodium lactate	84
				Sodium propionate	92
				Glycine	27
				N-methylglycine	68
				Ethylamine	100
2	6C3HED†	185	7.7	None	100
				α-Aminoisobutyrate	19
				β-Aminoisobutyrate	87
				L-Glutamate	35
				DL-α-Methylglutamate	91
				α-Ketoglutarate	100
				L-Aspartate	74
				L-Asparagine	26
				L-Lysine	57
				L-Arginine	29

*Molar concentration as L-amino acid was 20-fold that of azaserine.
†All cells harvested from 10- to 12-day-old tumors.
Cells incubated with azaserine and amino acids simultaneously at 37° C. for 20 minutes.

TABLE 3.—*Effect of glutamine on azaserine uptake by different ascites tumors*

Ratio (*I/S*)	Azaserine§ (*μmoles*)	Glutamine§ (*μmoles*)	Ascites tumors					
			70429/S*		70429/AZ-R-5†		DBA/2‡	
			$\frac{C_i}{C_e}$‖	Percent of control	$\frac{C_i}{C_e}$	Percent of control	$\frac{C_i}{C_e}$	Percent of control
	0. 5¶	0	31. 8	100	25. 1		11	100
	5. 0**	0	8. 0	100	5. 9	100	5. 4	100
1/5	0. 5	2. 5	9. 3	29			9. 1	83
	5. 0	25	1. 8	27	2. 7	46	1. 7	31
1/10	0. 5	5. 0	6. 1	19			5. 0	45
	5. 0	50	1. 3	20	2. 1	36	1. 2	22
1/20	0. 5	10	3. 0	9			3. 0	27
	5. 0	100	0. 8	15	1. 5	25	0. 6	10
1/50	0. 5	25	No zone	<3			<3. 0	<27
	5. 0	250	0. 6	12	0. 9	15	. 07	1

*Generation 60, harvested at 12 days.
†Generation 53, harvested at 17 days.
‡Generation 271, harvested at 12 days.
§Cells incubated with glutamine and azaserine simultaneously for 20 minutes at 37° C.
‖Distribution ratio.
¶57.5 μg./ml.
**575 μg./ml.

of that of the control, whereas 100 μmoles of glutamine were necessary to reduce the distribution ratio of strain 74029/AZ-R-5 to the same extent.

Discussion

The intracellular concentration of azaserine by 5 ascites tumors has been investigated *in vitro*. One of these is sensitive to azaserine *in vivo*, while the other 4 strains, 2 of which were derived from a sensitive line, are resistant. It appears that there is a quantitative difference in the extent of drug accumulation between the sensitive and resistant cell populations at low extracellular azaserine concentrations (10 μg./ml. and below). At higher extracellular concentrations the differences in uptake among the strains are less consistent, obscuring a clear differentiation.

Intracellular accumulation of azaserine is inhibited by the assimilatory inhibitors sodium azide and 2,4-dinitrophenol. These findings are quite similar to those of Jacquez with Ehrlich ascites carcinoma (*4*).

Azaserine uptake was antagonized and intracellularly accumulated azaserine was displaced by many amino acids. Methionine was among the most potent antagonists and displacement agents, the D-isomer being more active than the L-isomer. The superiority of D-methionine over its antipode indicates that it is functioning directly and not by racemization. No specific optical configuration is therefore necessary for displacement activity. In studies of amino acid uptake by bacterial cells, Cohen and Rickenberg found D-valine inactive in blocking the uptake of L-valine (*10*).

A free α-amino and carboxyl group are essential for blocking activity of the amino acids. Those derivatives resulting from *N*-methylation, acetylation, or α-methylation of dicarboxylic amino acids do not compete, or compete poorly, with azaserine uptake in our systems. Correspondingly, they do not compete with amino acid uptake in Ehrlich ascites cells nor are they accumulated by them (*11*). Although the uptake of amino acids has not been studied in our tumors, such parallelism indicates a common mechanism, at least in part, between azaserine and amino acid uptake. However, β-amino acids that are concentrated by the Ehrlich ascites cells (*11*) do not compete with azaserine uptake.

On the basis of these studies, it is clear that azaserine is accumulated intracellularly by the tumors under investigation. This event, which is energy-activated and presumably enzyme-dependent, is essential for drug action. Its magnitude could account for the differences in azaserine sensitivity in the tumors studied and the differences in inhibition of purine synthesis observed by Anderson *et al.* in strains 70429/S and 70429/AZ-R-1 (*12*). In contrast to these findings, a direct correlation between drug uptake and drug sensitivity could not be established for amethopterin action in *Streptococcus faecalis* cells (*13*). One amethopterin-resistant mutant accumulated 25 times more aminopterin than the amethopterin-sensitive parent strain.

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Spontaneous Leukemia at High Altitude in C58 Mice [1,2]

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Summary

A lower incidence of spontaneous leukemia was found in 329 C58 mice kept at high altitude than in 448 mice of the same strain kept at sea level. The difference was statistically significant. The amount of infiltration by leukemic cells and the weights of the localized tumors were greater in mice kept at sea level than in mice kept at high altitude. No difference in histology or cytology of the neoplasms could be detected. Leukemia occurred more often in female than in male mice, irrespective of altitude. A lower incidence was found in males kept at high altitude than in males kept at sea level, and the difference was statistically significant. The difference in the females was not so great but also was higher at sea level.—**J. Nat. Cancer Inst. 21: 985–997, 1958.**

The relation of cancer to high altitudes has not yet been adequately investigated under natural long-term conditions. Some isolated contributions in this field have appeared intermittently since the original work of Warburg in 1926 (*1*), Campbell and Cramer (*2*), Campbell (*3*), Sundstroem and Michaels (*4*), and Barach and Bickerman (*5*). All these studies were carried out at simulated high altitudes in pneumatic chambers at different levels of decompression or by the use of gas mixtures to lower the partial pressure of oxygen. We believe that simulated altitudes at pressures of 360 to 300 mm. for short periods, whether the exposure is intermittent or continuous, do not truly reproduce the conditions of natural acclimatization.

A study primarily concerned with physiologic changes and secondarily with pathologic alterations has been in progress at the Medical School of Lima since 1927. Human beings living permanently on high plateaus at levels between 12,000 and 15,000 feet and people and animals taken from sea level up to those altitudes, within a travel time of about 4 hours,

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have been the subjects of this investigation at the Institute of Andean Biology and Pathology. The Indians living for centuries in their natural habitat at high altitude have developed unusual physical characteristics, such as barrel chests and polycythemia. Some of these characteristics are well described and understandable as a response to the environment, but many facts about these Indians are probably still unknown and some information may be unreliable. Clinical experience indicates that people who live at sea level and those who live at high altitude differ in their biological response to various stimuli. Investigation of some of these differences has been recently attempted in the Institute of Andean Biology, but no experimental study has been made in respect to cancer. Although it is believed that the incidence of cancer in people living at high altitude is lower than at sea level, confirmatory data are lacking.

An extensive research program was designed to study the effect of natural high altitudes on neoplastic growth. This is being conducted in two laboratories: one at Morococha at 14,900 feet with a mean barometric pressure of 446 mm. Hg, and the other in Lima at sea level.

This report deals with the incidence and growth of spontaneous leukemia in strain C58 mice at high altitude and at sea level.

Method

Mice of the C58 high leukemic strain of MacDowell were used. The breeders from which our own colony was started were sent by Dr. Lloyd W. Law, of the National Cancer Institute, Bethesda, Maryland, U. S. A., and bred at sea level. The litters were divided at weaning into 2 groups; one group was kept at sea level in Lima, and the other was taken to the high-altitude laboratory in Morococha.

A total of 806 mice was distributed between the two laboratories. From these, 29 animals were not included in the final report (19 at high altitude and 10 from sea level) because they died before the age of 20 weeks. Spontaneous leukemia in these experiments never appeared earlier than 20 weeks of age. The total number of animals tabulated for the high-altitude group was 329, and 448 for the sea-level group. A greater number of animals was included in the sea-level group, and these were mainly females because it was necessary to keep an adequate number of them at sea level in order to breed and maintain the colony. Later an almost equal littermate distribution as well as sex distribution was maintained. More than 200 animals for each group were then about equally distributed.

Similar experimental conditions were established for both the low- and high-altitude groups of mice. The same temperature (78 to 80° F.) and humidity (65%) were maintained and the animals were fed the same standard diet, a modified Purina dog chow supplemented with 5 percent dried Brewer's Yeast and cod-liver oil. Water from the same source was supplied *ad libitum*. The animals were checked daily and weighed once a week and an attempt was made to maintain equal nutritional conditions in both groups. Complete autopsies were performed when some of the mice died; others were killed when near death and autopsied. At

autopsy the weights of certain organs and all tumors were always recorded. The tissues were fixed in Zenker's-acetic fluid, embedded in paraffin, and routinely stained with hematoxylin and eosin.

Results and Discussion

The Incidence of Leukemia

At high altitude only 173 of 329 mice developed leukemia (an incidence of 52.6%), whereas at sea level 324 of 448 mice developed leukemia (an incidence of 72.3%). The difference between these groups was 19.7 ± 3.6, which is a statistically significant lower incidence at high altitude. The results are summarized in table 1.

Age at Death from Leukemia

The age of the animals at death or killed when near death was recorded in weeks. The average age of the animals with leukemia in the high-altitude group was 55.2 ± 1.09. At sea level the average age of those with leukemia was 51.6 ± 0.85. The difference of means between both levels was 3.6 ± 1.20, which indicated that leukemia developed somewhat later at high altitude than at sea level. The comparative frequency of leukemia by age is shown in text-figure 1.

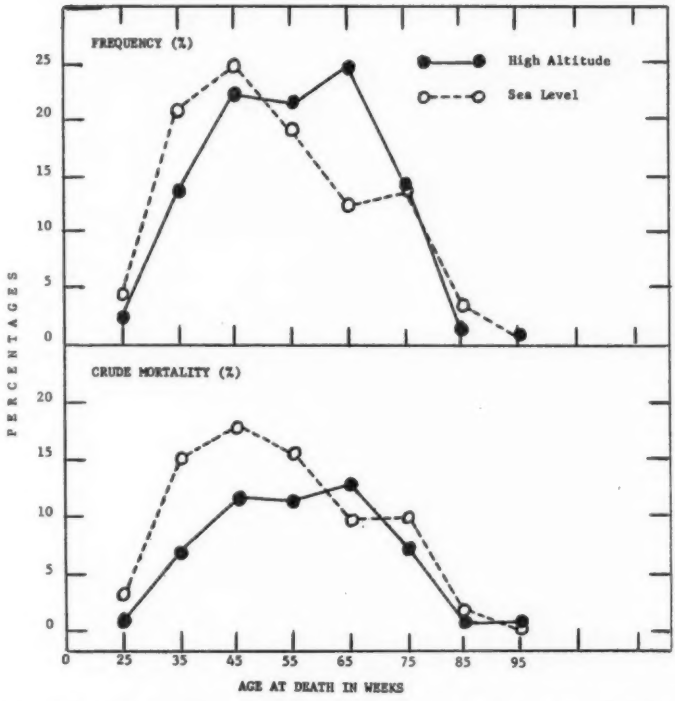
The diagram on top shows the percentage frequency and the diagram at the bottom shows the crude mortality rate of leukemia by age at both altitudes. The solid line indicates the results at high altitude and the broken line results at sea level. It clearly shows that the ascent line of percentage frequency at high altitude is below that at sea level with a shift to the right. The highest peak in the sea-level line is reached at an approximate mean age of 45 weeks and thereafter starts its descent until the end of experiments at a mean age of 90 weeks. The maximum percentage frequency at high altitude is reached at approximately a mean age of 65 weeks, and then a quick descent follows until the end. These findings and the statistical analysis of the data quite clearly indicate that leukemia develops at an earlier age at sea level than it does at high altitude. It was found that male mice whether leukemic or nonleukemic lived longer than female mice. The difference is significant at the 1 percent level.

Incidence of Leukemia with Regard to Sex

A total of 222 males and 107 females was studied at high altitude, while at sea level there were 214 males and 234 females. The disproportion between females at the two levels was explained previously. At both levels, males were less affected than females and lived longer, irrespective of morbidity. In male mice, a higher incidence of leukemia was found at sea level than at high altitudes. The difference amounted to 21.4 ± 4.6 percent. Less significant is the difference of 9.6 ± 5.3 between females, but again the higher incidence appeared in the sea-level group (table 2). Statistically, no clear-cut difference can be detected among females. Perhaps the excessive number of them at sea level somewhat substantiates

TABLE 1.—*Spontaneous leukemia at high altitude in strain C58 mice*

Experimental group	Number of mice	Leukemia		Percent	Mean age (*weeks*)	Mean weight (*gm.*)	
		Number	With			Prior to death	At death
High altitude (Morococha)	Male 222	122	100	45. 0	58. 0		
	Female 107	34	73	68. 0	51. 1		
	Total 329	156	173	52. 6	55. 2	24. 2 ± 0. 2	20. 9 ± 0. 2
Sea level (Lima)	Male 214	72	142	66. 4	53. 4		
	Female 234	52	182	77. 8	50. 2		
	Total 448	124	324	72. 3	51. 6	24. 8 ± 0. 1	23. 5 ± 0. 2
Differences				19. 7	3. 6	0. 6	2. 6



TEXT-FIGURE 1.—Percentage frequency of spontaneous leukemia in C58 mice by age (in weeks) at high altitude and at sea level.

the possibility that the total incidence might account for this uneven distribution. Notwithstanding, in estimating the percentage frequency of leukemia between females of both altitude levels, the difference actually is higher at sea level.

TABLE 2.—*Incidence of leukemia by age and sex*

Sex	High altitude		Sea level		Differences	
	Average age*	Percent	Average age*	Percent	Age*	Percent
Male	58. 0	45. 0	53. 4	66. 4	4. 6 ± 1. 6	21. 4 ± 4. 6
Female	51. 2	68. 2	50. 2	77. 8	0. 9 ± 1. 6	9. 6 ± 5. 3
Total	55. 2	52. 6	51. 6	72. 3	3. 6 ± 1. 2	19. 7 ± 3. 5

*At death of the animal.

Nutritional Condition at Sea Level and High Altitude

Care was taken to maintain the same nutritional conditions. The animals were weighed once a week and the weight just before autopsy was

also recorded. It was believed that the weight at death might not be a reliable indicator of the nutritional condition of the animals since the animals died or were killed because of illness. For this reason the weights of the mice recorded about 6 weeks prior to death were also tabulated in order to furnish a comparison between the 2 groups.

The average weights were calculated for the total group, as shown in table 3. The average weights of the total group, of the animals with leukemia 6 weeks prior to death, and of the nonleukemic animals were similar in both experimental groups, which indicated that the nutrition and body weight were well-maintained at both altitude levels. The average weights of both experimental groups at death indicated that a more significant loss of weight occurred at high altitude than at sea level. The difference of mean weights of leukemic mice 6 weeks prior to death and at death was 3.3 ± 0.3 at high altitude, while at sea level it was 1.3 ± 0.2.

TABLE 3.—*Average weights of mice*

Location	Total		Leukemic mice		Nonleukemic mice
	Prior to death	At death	Prior to death	At death	At death
High altitude	24.0 ± 0.1	20.8 ± 0.2	24.2 ± 0.2	20.9 ± 0.2	23.8 ± 0.2
Sea level	24.6 ± 0.1	23.1 ± 0.2	24.8 ± 0.1	23.5 ± 0.2	24.0 ± 2.3

Type and Extent of Anatomical Lesions

The leukemias were separated into localized or generalized groups, depending upon whether the lesions were in one area or were widespread. Each of the 2 major groups was further subdivided according to the predominant type of cell into lymphocytic, reticulum-cell, or plasma-cell neoplasms. A 3rd group was added to include neoplasms other than leukemia (table 4).

At high altitude 61 of the 173 leukemic mice had localized neoplasms (35.3 %), while the remaining 112 mice had generalized leukemia (64.7 %). At sea level 45 of the 324 tumor-bearing mice had localized neoplasms (13.9 %), while the remaining 279 mice had generalized leukemia or extensively involved leukemia (86.1 %). In the localized group at high altitude, the distribution according to the type of cell was 29.4 percent of lymphocytic neoplasms and 6.9 percent of reticulum-cell neoplasms. In the same group at sea level there were 13.3 percent lymphocytic tumors and 0.6 percent reticulum-cell neoplasms. At high altitude 50.9 percent of the generalized leukemias was of the lymphocytic type, 13.3 percent was reticulum-cell type of neoplasms, and 0.5 percent was plasma-cell neoplasms. One case of thymoma was found to be associated with lymphocytic leukemia at high altitude (figs. 1 through 6).

At sea level, 70 percent of the leukemias was the lymphocytic type, 15.7 percent the reticulum-cell type, and 0.4 percent was plasma-cell neoplasms.

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TABLE 4.—*Type and extent of lesions*

Experimental group	Localized neoplasms			Generalized leukemia		Average tumor weight (*gm.*)	Others		
	Type	Number	Percent	Number	Percent			Number	Percent
High altitude	Lymphocytic	51	29. 4	88	50. 9		Nonleukemic mice	8	
	Reticulum-cell	10	6. 9	23	13. 3		Leukemic mice	4	
	Plasma-cell	0	—	1	0. 5	3. 86			
Leukemic mice (173)	Total	61	35. 3	112	64. 7			12	3. 65
Sea level	Lymphocytic	43	13. 3	227	70. 0		Nonleukemic mice	5	
	Reticulum-cell	2	0. 6	51	15. 7		Leukemic mice	1	
	Plasma-cell	0		1	0. 4	4. 76	Nonleukemic mice	3	
Leukemic mice (324)	Total	45	13. 9	279	86. 1			9	2. 0
						Difference 0.90			

The extent of tumor growth and the degree of infiltration of several organs by leukemic cells were estimated by the weight of localized tumors of the thymus, mesenteric or subcutaneous lymph nodes, uterus, or ovaries, etc., in addition to the weights of the liver and spleen. These organs are frequently infiltrated by leukemic cells when hepatomegaly and splenomegaly of various degrees are present. The average weight of localized tumors and the liver and spleen was 3.86 gm. at high altitude, and 4.76 gm. at sea level, the difference being 0.90 gm.

A total of 12 mice at high altitude developed neoplasms other than leukemia. Four of these tumor-bearing mice (2 with lung tumors, 1 with a biliary-duct adenoma, and 1 with hepatoma) also had leukemia. The tumors in the other mice at high altitude were 5 lung tumors, 1 hepatoma, 1 squamous-cell carcinoma of the oral region, and 1 hemangioendothelioma of the testis. One of the lung tumors was unusually large, measuring 10 mm. in diameter with another small nodule in the opposite side of the lung. One instance of malignant adenoma of the lung infiltrating the chest wall and diaphragm was found also. At sea level 5 nonleukemic mice developed other tumors, *i.e.*, 4 lung tumors and 1 hepatoma. One of the leukemic mice at sea level also had hemangioendothelioma of the liver.

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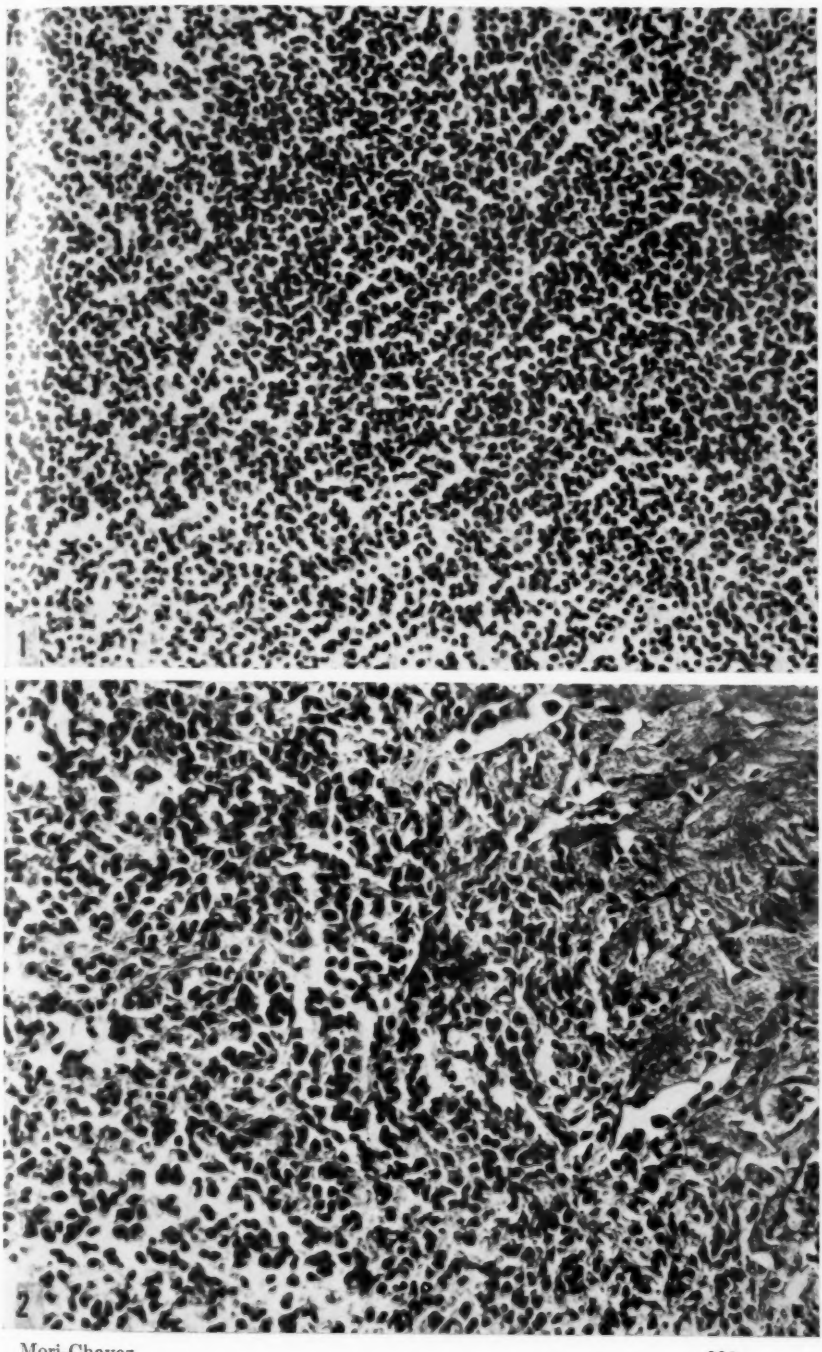
Plate 96

Figure 1.—Photomicrograph of a lymphocytic type of tumor of the mesenteric node. × 290

Figure 2.—Photomicrograph of a reticulum-cell neoplasm of the thymus, Type A. Neoplastic cells are infiltrating the heart muscle. × 290

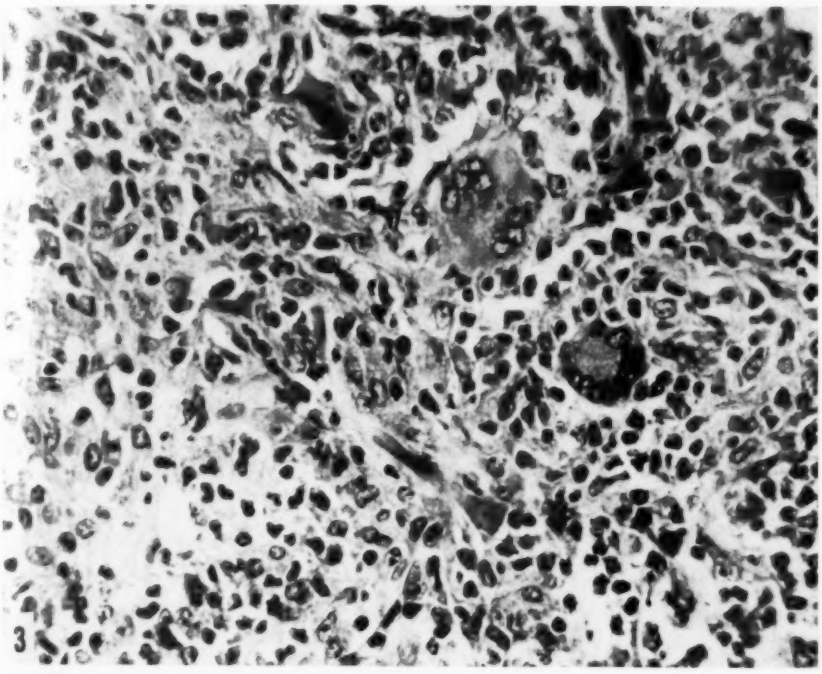


Mori-Chavez

PLATE 97

FIGURE 3.—Photomicrograph of a reticulum-cell neoplasm, Type A, with giant-cell formation that extensively infiltrated the liver. × 475

FIGURE 4.—Photomicrograph of a plasma-cell neoplasm of the spleen. × 830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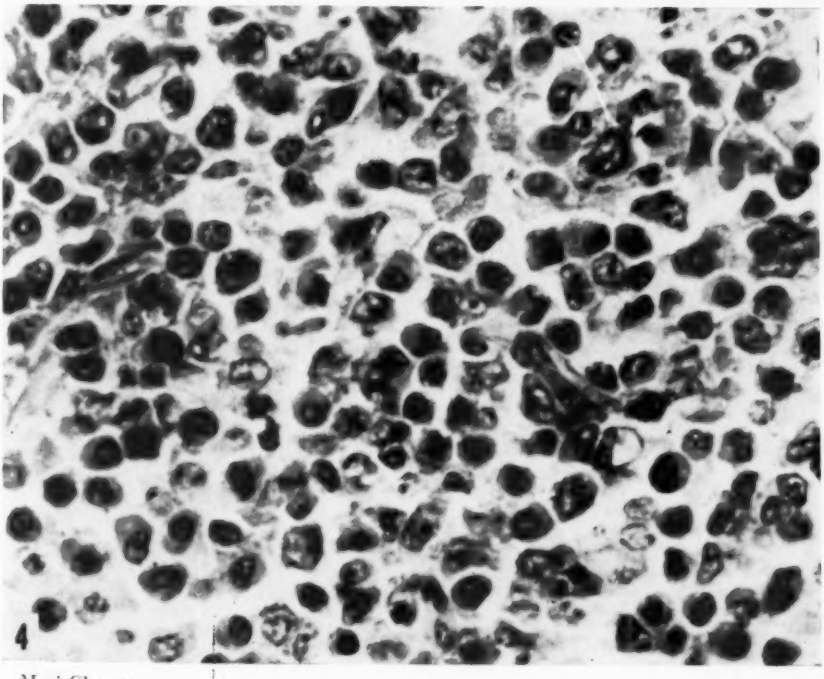
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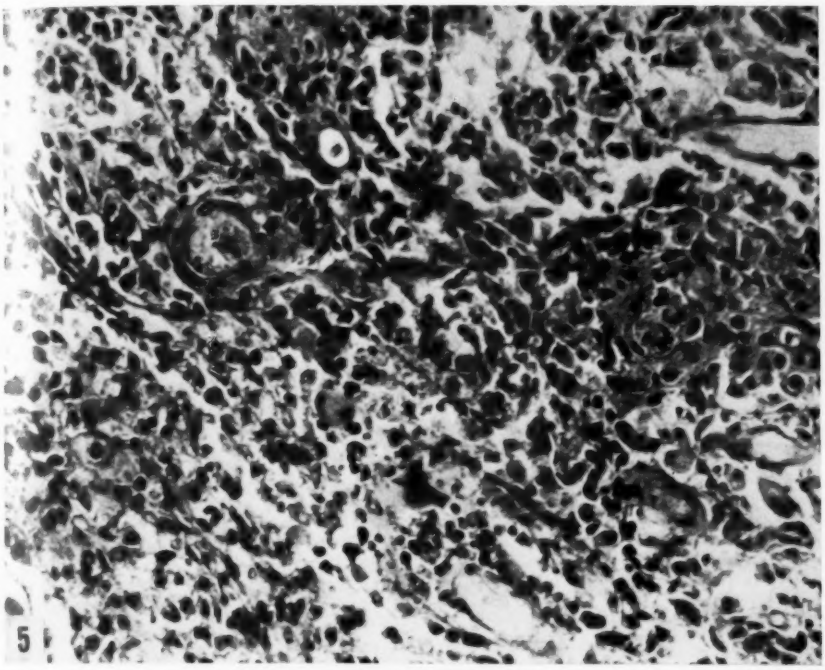
PLATE 98

FIGURE 5.—Photomicrograph of a thymoma. The whorl-like masses of reticulum cells resembling Hassall's corpuscles are clearly shown intermingled with lymphocytes. × 340

FIGURE 6.—Photomicrograph of a hemangioendothelioma of the testis. × 290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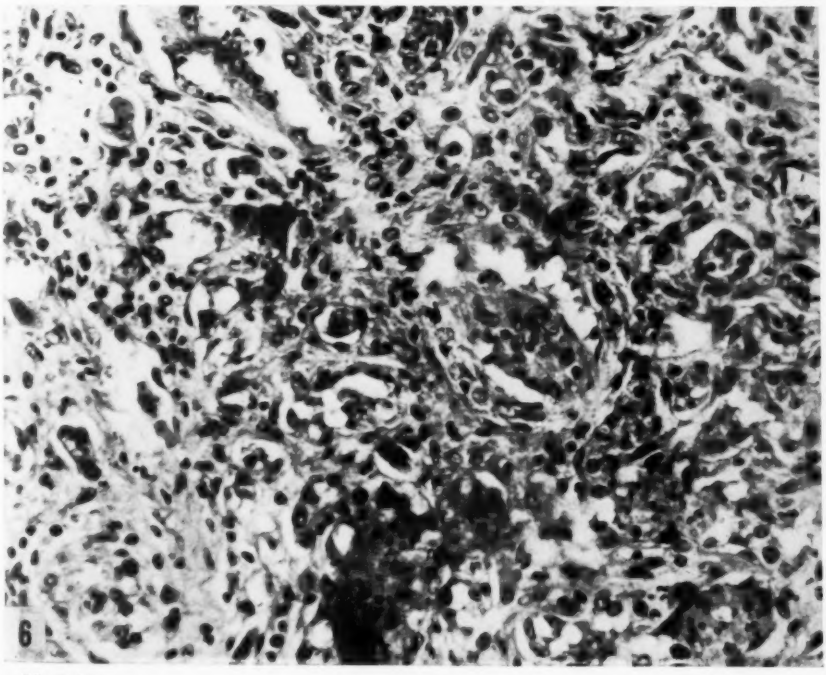
6

Histopathogenesis of Carcinoma Induced in the Glandular Stomach of C57BL Mice by the Intramural Injection of 20-Methylcholanthrene [1]

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Summary

A single dose of 0.3 mg. of 20-methylcholanthrene in an aqueous Methocel suspension was injected intramurally into the submucosa of the glandular stomach of 109 (effective number 108) strain C57BL mice. The experiment lasted 175 days. During this time mice were killed at regular intervals to afford material for a study of the histopathogenesis of carcinoma of the glandular stomach. Twenty-four of 50 mice killed in the effective tumor period had malignant gastric tumor with a carcinomatous element. Adenocarcinoma of the glandular stomach was diagnosed in a mouse killed on the 42nd day post injection and in 21 other mice necropsied between the 56th and 175th day. In addition, there was 1 mixed adenocarcinoma and sarcoma, 1 mixed adenoacanthoma and sarcoma, and 1 sarcoma. The results of this experiment compared with those of a somewhat similar but longer term experiment revealed a larger number of carcinomas in the present experiment. A possible explanation is that gastric neoplasms may fail to progress or may even regress after loss of methylcholanthrene from the site of injection in the wall of the stomach. As in experimental carcinomas of the forestomach and the small intestine, the histopathogenesis of induced carcinoma of the glandular stomach is a complicated process, participated in by the glandular mucous membrane of the stomach, the stroma, blood vessels, and muscularis. The experimental carcinomas of the glandular stomach appear to develop from multicentric neoplastic foci and to originate from the deep mucosa in association with umbilicate lesions.—**J. Nat. Cancer Inst. 21: 999–1035, 1958.**

Carcinomas, sarcomas, and tumors of mixed histologic type have been induced in the glandular stomach of mice of several strains by the intramural injection of carcinogenic polycyclic hydrocarbons (*1–3*). In one experiment (*1*), a comparison of the incidence of induced tumors of the glandular stomach in 6 strains of mice revealed that DBA mice were the most resistant to the carcinogen, whereas no great difference was found in the neoplastic response of strains BALB/c, C57BL, C3H, C3Hb, and A.

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Since the epithelial neoplasms seemed to develop more slowly in strain C57BL mice than in the other strains, and sarcoma of the stomach developed in fewer mice, this strain was chosen for a study of the histogenesis of carcinoma of the glandular stomach induced by a single intramural injection of 20-methylcholanthrene.[4]

Literature

Until recently the only known method for the induction of gastric adenocarcinoma was the injection of a carcinogenic hydrocarbon into the wall of the stomach (*4*). However, Nowell, Cole, and Ellis (*5*) have now reported the induction of adenocarcinomas and precancerous lesions of the glandular stomach of LAF_1 mice exposed to fast neutrons or to roentgen rays. The X-ray treatment was given as whole-body irradiation, but when supralethal doses were employed they were followed by injections of spleen homogenates for protection. In both groups of mice the gastric cancers were preceded by precancerous polypoid hyperplasia of the glandular stomach. The authors believed that the carcinogenic effect of the X rays was mediated through local, severe, and occlusive arteriosclerotic changes and that the fast neutrons exerted a direct carcinogenic effect on the gastric mucosa of the LAF_1 mice. When one considers the doses of radiation administered, however, this could hardly be the explanation, since Saxén (*6*) found no cancer of the glandular stomach of mice in which the gastric region had been exposed to equivalent or greater doses of X ray, even though in some animals the irradiation was followed by the oral administration of 9,10-dimethyl-1,2-benzanthracene.[5]

A fatal adenomatous lesion of the glandular stomach of mice exposed to neutrons from an atom bomb was described by Lushbaugh, Harris, and Storer (*7*). Many of the deaths were attributed to acute obstruction of the pylorus by polyps or to chronic gastric occlusion from the effects of diffuse adenomatous hyperplasia. The altered mucous membrane showed hyperplasia of the mucous neck cells and of indifferent cells, atrophy of parietal and zymogen cells, and intestinalization of some cells. There were some hyperplastic gastric glands which extended into the submucosal and subperitoneal connective tissue, usually following the course of blood and lymphatic vessels of the lesser curvature. Although the atypical adenomatous lesions of the stomach described by Lushbaugh and his associates (*7*) were similar to those described by Nowell and his associates (*5*), the former found no lesions that they were willing to diagnose as genuine carcinoma.

The occurrence of spontaneous adenocarcinoma of the stomach of laboratory animals was until recently so rare that no material was available for the study of its histogenesis.[6] Within the past year, however, Fortner (*8*) has reported the occurrence of gastric adenocarcinoma in the Syrian hamster, and Oettlé (*9*), in the African Mastomys. In a preliminary report of adenocarcinoma of the stomach of untreated hamsters and of hamsters that received subcutaneous injections of various substances which were considered unrelated to the gastric cancers, Fortner (*8*) mentioned, but did not describe, illustrate, or comment on the histogenesis of the lesion. The frequent association of the alimentary-tract tumors with lesions of the adrenogenital organs of the hamster suggested to him that a hormonal imbalance was the principal etiologic factor.

Oettlé (*9*) found a strikingly high incidence of spontaneous gastric cancer in the South African rodent, the Mastomys; an incidence greatly exceeding that of man. Oettlé, in his study of Mastomys living under laboratory conditions at the Medical Research Institute, Johannesburg, South Africa, calculated the stomach-cancer death rates and total mortality rates for Mastomys (natural deaths) of known ages at 6-month intervals. In his African colony, 98 of 236 Mastomys that died of natural causes had

[4] *Chemical Abstracts'* nomenclature: 3-methylcholanthrene.

[5] *Chemical Abstracts'* nomenclature: 7,12-dimethylbenz[*a*]anthracene.

[6] Hackmann (Hackmann Chr. Zbl. allg. Path. path. Anat. 91: 317–325, 1954) reported adenocarcinoma of the glandular stomach in 29 of 1,135 mice, but his only illustration (fig. 8) of a stomach with this lesion is not convincing.

cancer of the stomach. The youngest Mastomys with a gastric cancer was 6 months of age, while the oldest was 3½ years of age. The rate percent of cancer, which in some age groups exceeded 60 percent, was greater in female than in male Mastomys. According to Oettlé (*9*), many of these gastric cancers metastasized to the regional lymph nodes, liver, and lungs, and a few to remote sites, such as the pancreas, spleen, diaphragm, uterus, and adrenal gland. In the region of almost all gastric tumors, as well as in some stomachs with the earliest intramucosal stage of carcinoma *in situ* or even without detectable carcinoma, the mucosa of the fundic region showed hyperplasia. This mucosal hyperplasia was characterized by prominence of the longitudinal rugae, often in association with superficial erosions and gastric hemorrhages, but without chronic peptic ulceration. Focal gastric hypertrophy produced irregular mucosal nodules that sometimes consisted of multiple tiny elevations which imparted a cobblestone appearance to the mucosa. Gastric polyps were rare. Foci of lymphocytes were often present in the underlying submucosa, usually at the base of a mucosal fold. Gastric glands were occasionally present in the submucosa, but these ectopic glands did not appear to give rise to cancer. The surface cells of the mucosa were taller and the cytoplasm was more basophilic than that normally found. Secretory activity was minimal, although the secreted mucin, when present, retained its normal staining capacity. There were pseudostratification and proliferation of surface cells and cyst-like dilatation of the gastric foveolae. The mucous neck cells increased in number and extended to depths where normally they are not encountered, thus producing an alteration in the proportions of cell types in the gastric mucosa. Parietal and zymogenic cells were occasionally found among the mucous neck cells as were foci of undifferentiated cells with acidophilic cytoplasm, which were considered to be cell collections of early gastric carcinoma. There was branching of gastric glands, and an increased number of mitotic figures in the isthmic regions. Intestinal metaplasia of the gastric mucosa was not observed in the Mastomys, and atrophic changes, although not a striking feature, were occasionally seen in regions of submucosal invasion. The cardiac glands were occasionally hyperplastic, but did not appear to be precancerous.

It is difficult to refrain from overemphasizing the importance of Oettlé's discovery that Mastomys have a high incidence of spontaneous cancer of the glandular stomach. Although few investigators in this country are acquainted with the Mastomys, the animal may now become more extensively used as an experimental tool in the study of gastric cancer. Goodwin (*10*) has described the Mastomys as the great mother of rodents. It is a close relative of the common rat. It is called a multimammate mouse (*Rattus* [Mastomys] *natalensis;* A. Smith, 1834, syn. *Mastomys coucha*) because the female usually possesses at least 8 pairs and sometimes up to 12 pairs of mammae. On the average, a Mastomys may rear from 12 to 20 young, so that under favorable conditions the animal multiplies rapidly. The Mastomys is a buffy-brown rodent found in most parts of the continent of Africa. It lives by preference on cultivated land, where it does great damage to the grain crops. The Mastomys is also a transmitter of disease, for it carries bubonic plague into human dwellings in areas where that disease is prevalent.

In the rat, induced adenocarcinoma of the stomach, which follows the intramural injection of a carcinogenic hydrocarbon, is preceded by a precancerous lesion—the so-called "adenomatous diverticulum" (*11*). This lesion has no counterpart in other laboratory animals or in human beings. The diverticulum-like mass develops in continuity with the lumen of the stomach, which it may, in time, exceed in size. On section, the adenomatous diverticulum is riddled with microcysts and macrocysts that contain polypoid adenomatous nodules, mucus, inflammatory exudate, and necrotic material. The polypoid adenomatous nodules are composed of fibro-inflammatory tissue and epithelial cells, chiefly of the mucous variety, though foci of squamous metaplasia are present in some specimens of stomach. The development of the adenomatous diverticulum is accompanied by thinning of the stomach wall, but the integrity of the serosa and in part of the muscularis propria is usually maintained. The most deeply placed glands are separated from the surrounding viscera by a thick barrier consisting of fibrous inflammatory tissue and peritoneum, in which there are

remnants of the muscularis of the stomach. Fibrinoid degeneration appears in the stroma and in portions of the wall of many of the arteries; some arteries show chronic arteritis and partial or complete occlusion.

Howes and de Oliviera (*12*) described round-cell infiltration and edema that appeared around methylcholanthrene-impregnated threads almost immediately after implantation between the serosa and the acid-secreting mucosa of the stomach of rats. These changes were soon followed by ulceration and destruction of the acid and pepsin cells. Two weeks after the insertion of the thread, the mucous cells that survived relined the cleft and proliferated along the thread, creating a sinus that communicated with the lumen of the stomach. The cells lining the cleft formed acini, and sometimes these cells proliferated to form adenomatous lesions that became surrounded by newly formed reticulum. Later, this reticular barrier was penetrated by the aggressively proliferating glandular mucosa, which also infiltrated the surrounding tissues. Grant and Ivy (*13*), applying somewhat similar experimental procedures to the stomach of rats, observed the replacement of chief and parietal cells by mucous cells, the dilation of tubules, the formation of cysts, and the development—especially at the base of the gastric glands—of foci of immature and mature mucous cells and undifferentiated epithelial cells with numerous mitotic figures.

Stewart and Hare (*14*) tested the response of two areas of the glandular stomach of the rat to methylcholanthrene and found that the antral area is more susceptible to the induction of carcinoma and the adenomatous diverticulum than the acid-secreting fundic area near the greater curvature. In man, too, the majority of spontaneous carcinomas of the stomach arise in the antrum (*15–17*). These observations offer a basis for comparison of the two species.

In man, three forms of preinvasive carcinoma of the stomach have been described (*4*): 1) a *polyp* that develops cytologic alterations; 2) a nonpolypoid lesion very prone to ulceration, which occurs in the prepyloric region; and 3) the so-called "carcinoma *in situ*," a lesion composed of atypical cells and atypical mitotic figures widely distributed in the area of involvement but usually confined to the mucous membrane. Considerable difference of opinion has been expressed regarding the significance of the role of gastric ulcer, chronic gastritis, and intestinalization of the gastric mucosa in the etiology and pathogenesis of gastric cancer in man. Willis (*18*) conceives of carcinoma not as the product of spread from a single cell or a solitary focus but as a lesion that arises simultaneously or successively at several points over a relatively large area, the so-called "field effect." Human gastric carcinoma has been frequently described as multicentric in origin, and indeed it may be widespread over the entire gastric mucosa (*19*).

Materials and Methods

A total of 109 C57BL mice (effective number 108), 2 to 4 months of age, were used. All but 7 were male mice. There were, in addition, 6 untreated control mice killed at 3½ months of age.

The technique for injecting the carcinogen into the wall of the stomach was the same as that employed in a previous experiment (*1*). The mice were anesthetized by an intraperitoneal injection of sodium pentobarbital (Nembutal). The stomach was delivered through a midline incision in the epigastric region. With a 1 cc. tuberculin syringe fitted with a 27-gauge needle, 0.01 cc. of a suspension containing 0.3 mg. of 20-methylcholanthrene was injected into the anterior wall of the gastric antrum. A small yellow bleb in the tissue indicated a successful injection. The peritoneum and skin were closed by silk-thread suture. The methylcholanthrene-Methocel (methyl cellulose) suspension was not a permanently stable suspension, and sedimentation was a constant hazard. Therefore, to insure the injection of a uniform amount of methyl-

cholanthrene, the container was always shaken vigorously before loading the syringe, and the latter was always rotated rapidly just prior to insertion of the needle into the wall of the stomach. Every possible effort was made to deposit the carcinogen in the submucosa. In both experiments the injections of the methylcholanthrene-Methocel suspension were made by the same individual. The suspension used in the two experiments was all from one batch; it was prepared by the late Dr. Egon Lorenz.

The mice were individually numbered at the time of injection and caged in groups of 5 to 10. The animals were given pellets of Purina dog chow and tap water *ad libitum*. One mouse was killed at 1 minute post injection, and 1 each hour thereafter for 24 hours; 2 or more mice were killed daily during the 1st week, and either daily or every 2 days during the 2nd week. Subsequently, 1 or more mice were killed weekly or biweekly from 19 to 175 days (table 1). The 7 female mice were necropsied as follows: 1 at 70 days and 1 at 168 days; 2 at 175 days; and 3 at 161 days. All mice were killed with ether except 1 that died on the 83rd day. This mouse showed insignificant *post-mortem* changes and so was included in the experiment. One mouse found dead on the 34th day was discarded because of *post-mortem* change. This left the effective number of experimental mice at 108.

The stomach was always given first attention at necropsy. Approximately 0.5 ml. of Zenker's acetic-acid fluid was injected into the lumen of the stomach *in situ* after the esophagus and duodenum had been ligated close to the stomach. The stomach was then removed *in toto* and dropped into the fixative. A complete necropsy was performed on all animals. After the stomach was fixed and washed in running tap water, the ligatured ends, most of the forestomach, and, in some cases, a portion of the uninvolved glandular stomach were trimmed away. The injection site and surrounding area were left intact; this portion of the stomach was then embedded in paraffin and cut serially in sections 6μ thick. The entire block was cut and the sections were mounted in sequence on a series of glass slides. Alternate slides were colored with hematoxylin and eosin or by the periodic acid-Schiff reaction, except for occasional slides that were colored with Wilder's silver.

Definitions and Results

The terms "adenocarcinoma" and "adenoacanthoma" as applied to the gastric lesions listed in table 1 are used in this paper to designate epithelial neoplasms that invaded all layers of the stomach and penetrated the serosa. The terms "precancer" and "precarcinoma" are used in a broad sense to designate neoplastic-like lesions that varied in extent from minimal atypicality of glands in the mucosa to a more advanced process characterized by neoplastic invasion of the wall of the stomach, which, however, stopped short of penetration of the serosa. This rather arbitrary rule was adopted to make sure that atypical glandular proliferations, which are not uncommon in the stomach of some strains of mice, should not be mistakenly listed as malignant tumors. The term "sarcoma" is used

TABLE 1.—*Schedule of necropsies of C57BL mice: days post injection and number and types of tumors found in present experiment*

Days post injection..	1–28	42	56	70	79–83	84	91	98	105	106	112	120	126	133	140	149	154	161–168	175	Totals
Number of mice.....	58	2	2	2	2	2	3	4	1	2	3	3	3	3	2	3	3	7	3	108
Adenocarcinoma.....		1	2	1		2	1	3		1	3	2		2	1	1	1		1	22
Mixed adenocarcinoma and sarcoma....												1								1
Mixed adenoacanthoma and sarcoma....													1							1
Sarcoma.....									1											1
Total tumors.....																				25

TABLE 2.—*Schedule of necropsies of C57BL mice: days post injection and number and types of tumors found in earlier experiment (Stewart, Hare, and Bennett, 1953)**

Days post injection	16–58	90	91–119	124	161	170	173	174–204	205	212	215	220	226	232–344	347	348–465	505	513–563	584	589–662	Totals
Number of mice	7	1	6	1	1	1	1	2	1	1	1	3	1	33	1	39	1	6	1	3	111
Adenocarcinoma				1					1												2
Adenoacanthoma																	1				1
Mixed adenocarcinoma and sarcoma		1			1						1				1				1		5
Mixed adenoacanthoma and sarcoma							1														1
Sarcoma													1								1
Total tumors																					10

*This table was compiled from a segment of the previous experiment in which the reaction of the glandular stomach to the intramural injection of 0.3 mg. methylcholanthrene was tested in 6 strains of mice. All animals shown in this table are C57BL mice approximately equally divided as to sex. The similarities and differences between experiments depicted in tables 1 and 2 are given in the text.

to designate the tumors of connective-tissue type. The term "mixed tumor" means a combination of adenocarcinomatous or of adenoacanthomatous and sarcomatous tissue.

On the basis of these definitions, adenocarcinoma of the glandular stomach was diagnosed in a mouse killed on the 42nd day post injection and in 21 other mice necropsied between the 56th and the 175th days of the experiment. In addition, there was 1 mixed adenocarcinoma and sarcoma in a mouse necropsied at 120 days post injection, 1 mixed adenoacanthoma and sarcoma at 126 days, and 1 sarcoma at 105 days. The stomachs of all other mice examined showed changes, described hereinafter, that could be related to the effects of the injected carcinogen. The lesions in the antral mucosa of the stomach of the mice killed during the first 24 hours or during the first few days of the experiment were minimal compared to those lesions seen in mice killed 1 week or more following the injection. The gastric lesions in all mice that did not have carcinomas or sarcomas, by definition—even those killed during the first 24 hours and the first few days of the experiment—are listed in the broad group of precancerous lesions, in spite of the fact that the magnitude of the lesions differed considerably. Of the 7 female mice, 6 had a precancerous lesion, and 1 mouse that was killed on the 175th day had adenocarcinoma.

Gross Necropsy Findings

The site of injection of the methylcholanthrene-Methocel suspension in the wall of the antrum of the stomach was marked by the presence of a small, round, yellow bleb, 1 to 3 mm. in diameter. This bleb was observed in the mouse necropsied 1 minute after the injection procedure, and was recognized in the majority of the mice necropsied during the first 19 days of the experiment. Thereafter, this focus was obscured by local thickening of the tissues and by adhesions, or it was absent because the injected material had disappeared. Fibrous adhesions emanating from the region of the injection site were first observed in 2 mice necropsied 4 and 6 days post injection, and, although the adhesions varied considerably in extent in different animals, they were present in the majority of mice necropsied 1 month or more after surgery. The adhesions extended from the glandular stomach to the liver, anterior abdominal wall, small intestine, omentum, spleen, diaphragm, or forestomach. A few animals with extensive peritoneal adhesions also had peritonitis, peritoneal abscess, and pancreatitis. The gastric antrum was palpably thickened in 2 animals at 13 days and in 2 animals at 42 days after injection, and one of the latter had a carcinoma on histologic examination. From 56 to 91 days, palpable thickening of the antrum was noted fairly frequently, and, although the thickening was described grossly as resembling scar tissue, a number of these lesions proved to be carcinoma on subsequent histologic examination. The gastric antrum of many mice necropsied from 98 days to the completion of the experiment was grooved by an annular constriction resembling a napkin-ring deformity, which caused some

degree of pyloric obstruction with resultant dilatation of the stomach. In several mice necropsied 120 days or more following injection of the carcinogen, the stomach was found to be kinked, angulated, and rotated, thus causing the esophagus to be drawn down into the peritoneal cavity. This retraction seemed to be due to the combined effect of the napkin-ring deformity, pyloric obstruction, secondary dilatation of the stomach, and adhesions.

The examination of the mucosal surface of the antrum was not as complete as might be desired because *in-situ* fixation and *en-bloc* excision of the stomach limited inspection of the antral mucosa to a view through the open ends of the trimmed specimen. However, even with this limitation, some interesting observations were made. At 56 days post injection, the glandular stomach of 1 mouse showed an elevated, thickened mucosal area at the site of injection that proved histologically to be a carcinoma. In another mouse killed at 70 days, a thickened mucosal area of similar appearance was found to be a precancerous lesion upon histologic examination Thickened mucosal areas noted in several specimens examined between 70 and 100 days were either precancerous lesions or carcinomas. A specimen obtained at 105 days post injection showed a neoplasm (histologically, a sarcoma) that extended from a point proximal to the limiting ridge to the pyloric ring. This tumor infiltrated the liver and pancreas and, while covered by intact gastric mucosa, projected into and filled a large portion of the lumen of the stomach. A 4 mm. thickened area on the mucosa of a gastric specimen observed at 106 days proved histologically to be a precancerous lesion, whereas a 2 mm. slitlike depression in a thickened area of mucosa in a specimen observed at 133 days was found to be a carcinoma.

Other lesions observed one or more times in the mice necropsied in this experiment were enlargement of the spleen and thymus gland, sinusoidal dilatation of the regional lymph nodes, pneumonia, bronchiectasis, ascites, pericarditis, nephritis, hydrocephalus, hepatic abscess, pigmentation of the spleen, polyps of the duodenum, amyloidosis, primary pulmonary tumors, thrombosis of the portal vein and mesenteric vessels, periarteritis of the mesentery, and chronic pancreatitis.

Microscopic Observations

Day 1.—One mouse was killed 1 minute after injection of the carcinogen and 1 mouse every hour thereafter, thus making 25 gastric specimens available for study during the first 24-hour period. The methylcholanthrene that had been injected into the wall of the antrum was dissolved in the solvents used in the process of paraffin embedding, and no crystals of methylcholanthrene were seen in the stained sections. In the specimens examined during the first 6 hours, the connective-tissue fibers at the site where the injection had been made were separated by the methylcholanthrene-Methocel suspension. By 7 hours, acicular spaces from which precipitated methylcholanthrene crystals had been dissolved were

observed. Some evidence of inflammatory change was present in all specimens. An acute inflammatory exudate, confined at first to the submucosa, quickly spread to the mucosa, the muscularis, and the necrotic tissue around the needle tract. In some specimens the distal two thirds of the glandular stomach was involved, but the exudate, as if restrained by a mechanical barrier, did not extend beyond the pyloric ring. The inflammatory process was characterized by hyperemia and engorged lymphatic vessels and by the presence of edema fluid, blood, fibrin, granulocytes, lymphocytes, and histiocytes. The granulocytes, many of them degenerated, predominated and were often congregated in round or linear foci. Many histiocytes contained engulfed acidophilic debris. Fibrinoid degeneration first appeared in collagenous connective tissue at 10 hours, and in the wall of an artery at 15 hours. By 24 hours an area of necrosis had formed in the center of the submucosal inflammatory focus and had extended into the adjacent muscularis. Around the periphery of the inflammatory focus, young fibroblasts and capillaries had proliferated.

While these changes were in progress immediately around the injection site, various alterations were taking place in the overlying mucosa. At 2 hours post injection, the mucosa was slightly thickened by distention of the basal glands [7] with mucus and by edema of the superficial lamina propria. Between 4 and 10 hours the reticular basement membrane fragmented and fell away from many of the basal glands in the lamina propria. There appeared to be a loss of nuclei from some of the cells lining these glands. Between 11 and 14 hours the mucus that distended the fovea and the basal glands stained faintly; some of the basal glands were lined with epithelium that was 2 or 3 cells in thickness, indicating cellular proliferation following injury by the carcinogen. Glands in the cardiac region of the stomach and Brunner's glands of the duodenum, although remote from the site of injection, were also slightly dilated. At 24 hours the thickening of the mucous membrane above the injection site was further accentuated by the contraction of the underlying muscularis propria. The cells of the midmucosal glands of this area were hyperchromatic, slightly enlarged, increased in number, and disarranged in their polarity.

Mitotic figures in the mucosal epithelium were usually decreased in number during the first 10 hours of the experimental period, and in some specimens there were fewer of them in the basal-gland cells than in the neck cells. After 10 hours, mitotic figures were more numerous, especially at the junction of the lower one third and the upper two thirds of the mucosa. Some cells in mitosis appeared necrotic. Mitotic activity continued to increase and reached a peak at 14 and 15 hours post injection, after which there was an abrupt decrease so that at 16 hours there were fewer mitotic figures than at any previous period. This low mitotic activity persisted throughout the remainder of the first 24-hour period. Most of the mitotic figures were normal in appearance, but, in one speci-

[7] The term "basal gland" as used throughout this paper refers to the base or the blind end of the gland.

men at 9 hours, bizarre figures in association with a few cells with hyperchromatic nuclei were seen.

Days 2 to 9.—The inflammatory cell infiltrate increased in extent around the site of injection of the carcinogen, and the area of necrosis enlarged during this period (fig. 1). There were many acicular spaces which, individually, were surrounded by a cuff of neutrophils or eosinophils (fig. 2). Many of the numerous macrophages around the central degenerated area contained acicular spaces and periodic acid-Schiff positive material. The surrounding edematous connective tissue showed fibrinoid degeneration, and many individual fibroblasts were pyknotic (fig. 3). Some venules and arterioles were dilated, their endothelium was proliferated, and their walls showed fibrinoid degeneration. In 4 specimens, the first at 5 days, foci of newly proliferated, abnormal, spindle-shaped cells, either fibroblasts or smooth muscle cells, were suggestive of the earliest stage of the formation of sarcomatous tissue (fig. 4). At the periphery of some of these foci there was a richly vascular tissue showing perivascular fibrosis and atypism of ganglion cells.

The first umbilicate lesion appeared in the thickened mucous membrane of the glandular stomach of an animal killed 3 days post injection. This early umbilicate lesion was characterized by a small, saucer-shaped depression in the mucosa, the result of atrophy, erosion, and depletion of mucus in the superficial portion of the gastric glands. The deep portion of these same glands contained individual epithelial cells that varied in size, shape, and polarity. *The first evidence of neoplastic infiltration of a gland into the submucosa* was noted in a specimen of stomach examined 4 days post injection. This specimen revealed one large and several small umbilicate lesions, the small lesions being only 2 to 4 glands in width. A basal gland in the deep portion of one of the umbilicate lesions was closely applied to the muscularis mucosae, and another gland had extended into the submucosa along a path adjacent to an artery where there was a natural interruption of the muscularis mucosae. Umbilicate lesions were present in the glandular stomach of 2 of 4 mice necropsied 5 days post injection, and these lesions showed similar changes to those described above (figs. 5, 6, and 7). In one of these specimens there was an area of necrosis, probably an infarct, involving the full thickness of the gastric mucosa and a corresponding segment of the muscularis mucosae.

From 6 to 9 days post injection, 7 of the 10 glandular stomachs examined showed one or more umbilicate lesions (fig. 8). There was a greater degree of thickening of the mucosa above the injection site than in specimens examined previously. The umbilicate lesions were larger than those noted earlier and showed, in addition to the features described, other changes indicative of the early evolution of neoplastic growth. The glands composing the umbilicate lesions were irregular in size and contour and frequently atrophic and depleted of mucus, but occasionally dilated. The lining cells of some of the glands were alternately tall and flat, and this irregularity in the height of the cells imparted a saw-tooth appearance to the outline of the lumen. Other glands were lined with cells that were

all flattened or all pseudostratified, and not all nuclei occupied a basal position within the cell. There were also solid nests of cells. The number of mitotic figures varied from area to area; as many as 6 to 10 might be seen in one high-power field. The border of the umbilicate lesion was surrounded by overhanging, hyperplastic, mucosal glands, beneath which the smooth muscle cells of the muscularis mucosae were reoriented to point upwards in the direction of the hyperplastic mucosa. Some basal glands, with lumens partly or completely obliterated by stratified epithelial cells, were flattened out against the muscularis mucosae and elongated laterally. Small buds branched off the deepest aspect of many of these glands. In one specimen the base of the mucosa, the muscularis mucosae, and the muscularis propria were adherent to one another.

Even in the absence of umbilicate lesions or in areas of mucosa not involved by them, changes in the direction of neoplasia occurred in the thickened mucous membrane during this 2- to 9-day period. The mucosa was consistently thickened and elevated immediately above the injection site, and mucus was most abundant in its superficial one third. The basal glands, which were either filled with or depleted of mucus, were pressed tightly against the muscularis mucosae. At the lateral extremities of the thickened area in some specimens, the basal glands, 2 or 3 in width, had penetrated into the submucosa through an apparently otherwise healthy muscularis mucosae. Only rarely was the border of the muscularis mucosae around the infiltrated glands necrotic. Within the submucosa, the dilated, branched, and complicated infiltrated glands were devoid of any demonstrable reticular basement membrane but were surrounded by pale, plump, atypical fibroblasts. The lumens of such glands, as seen in serial sections, remained in continuity with the corresponding glands of the mucosa, which in turn opened onto the surface of the stomach through widely dilated fovea. The deepest aspect of some of the infiltrated glands was frequently not intact but, instead, appeared to be turned inside out like the sleeve of a coat. The epithelial cells of such defective glands faced in the direction of the stroma instead of in the direction of the lumen. The lining cells of the intact glands were of three types: 1) tall, hyperchromatic, mucous cells in which the droplets of mucus were finely divided; 2) greatly elongated, mucus-free cells, possibly newly regenerated cells or cells that had been subjected to stretching; and 3) large, pale cells of unusual morphology. The large, pale cell had a prominent, often hyperchromatic, nucleus and a good deal of cytoplasm, which contained a single vacuole and numerous fine granules that were brown in the hematoxylin-eosin preparation (fig. 9). While this cell could not be positively identified, the possibility was considered that it represented a cell intermediate in type between glandular epithelium and squamous epithelium.

Days 11 to 28.—The changes in and around the injection site in the submucosa differed in certain respects from the changes noted at earlier periods. There were many more telangiectatic and thrombosed veins. The fibroblasts had increased in number and some were degenerated;

those that were viable had small nuclei and short processes or large hyperchromatic nuclei and thick processes. Fibrinoid degeneration was especially prominent in the connective-tissue stroma of areas infiltrated by glands and in the walls of blood vessels, some of which showed proliferation of endothelial cells. There was a concentration of inflammatory cells and fibrous tissue around the infiltrated glands. Acicular spaces were present in some, but not all, specimens.

The mucous membrane exhibited progressively increasing signs of neoplastic change during this period. It was plaquelike and 3 to 5 times thicker than normal. The plaque might be confined to the antrum or might reach to the acid-secreting area, depending somewhat upon the extent of the underlying submucosal inflammatory process. Foci of atypical glands were much more numerous than heretofore, and 3 to 4 mitotic figures could be found in each gland. One or more tiny or large umbilicate lesions studded the thickened mucous membrane. Many of them were significantly depressed below the surface level of the mucosa, and some were cryptlike, with the margins bordered by overhanging hyperplastic glands.

Many basal glands were dilated and some were branched, and the long axis was oriented in various directions. Some were filled with mucus and others were empty. Individual basal glands were separated from the basement membrane by edema fluid and inflammatory cells, and the deep portion of such glands was denuded of epithelial cells. Some glands were destroyed, and the cells of others exhibited pseudostratification, hyperchromasia, and an increase in the number of mitotic figures. The glands that impinged upon the muscularis mucosae usually retained their lining cells and basement membrane and were often flattened and T-shaped, as though they had met resistance in their growth toward the submucosa (fig. 10). There was usually no distortion of the muscularis mucosae, however, indicating that either the impinging glands exerted no pressure or that, if they did, it was insufficient to deform this muscular coat. At this period, while it was generally true that pictures like those described were associated with single or multiple points of glandular penetration of the muscularis mucosae (fig. 11), such was not always the case. This discrepancy emphasizes the difference in the tempo of the neoplastic process in different animals of the same species and strain, all treated with exactly the same dose of carcinogen at the same time.

The deep portion of the umbilicate lesions usually marked the site of single or multiple (from 3 to 12) points of glandular penetration through the muscularis muscosae into the submucosa. There was usually a clean break in the muscularis mucosae at points through which the glands had penetrated. Where the glands had infiltrated the muscularis mucosae along the course of blood vessels, the walls of these vessels and the surrounding connective tissue frequently showed fibrinoid degeneration and inflammatory changes. Rarely there was a suggestion that the glands had penetrated the submucosa by way of perivascular lymphatic vessels. The proliferated glands within the submucosa still maintained their

continuity with the parent mucosal gland as proved by examination of serial sections. Such clusters of glands in the submucosa were sometimes not greater in width than the thickness of a few sections, whereas others occupied the space of numerous consecutive serial sections (figs. 12 and 13). Foci of proliferated glands in the submucosa often remained separated from each other by intervening wedges of the submucosal connective tissue. Mitotic figures appeared to be less numerous in the glands of the submucosa than in those of the mucosa. A deep, cryptlike umbilicate lesion in a 28-day specimen was associated with two sets of blood vessels that had homogeneous, deeply acidophilic walls and lumens narrowed by proliferated endothelial cells. It is probable that the blood supply to the area was thus compromised, and that this accounted for the cryptlike lesion. One area of the submucosa of this 28-day specimen showed a peculiar type of edema with much poorly stained fibrillar material interspersed with bare nuclei of fibroblasts.

Day 42.—In the glandular stomach of 1 of the 2 mice killed, neoplastic glands had invaded all coats of the wall of the stomach and had extended through and onto the peritoneum. This lesion was classified as the first definitive carcinoma. An umbilicate lesion in the thickened mucous membrane was continuous with the infiltrated nodule of carcinoma. The deeply placed glands of the carcinoma contained faintly colored mucus and degenerated and desquamated cells. A few glands showed intestinalization of epithelium and squamous metaplasia. The reticulum around the tumor cells appeared healthy. A plaque of fibrous tissue had formed around the neoplastic nodule on the serosa.

The glandular stomach of the 2nd mouse killed at 42 days post injection revealed neoplastic changes that fell short of fulfilling the criteria for carcinoma. The mucosa was eroded above the site of injection. The submucosa and muscularis propria contained a large, dilated, papillary, intercommunicating glandular structure. In both of the 42-day specimens there were telangiectatic, occasionally thrombosed, blood vessels, and patchy, often segmental, areas of eosinophilic degeneration in the outer coats of the blood vessels and in the perivascular connective tissue. Fibrinoid degeneration was present in the submucosa and peritoneal plaque.

Day 56.—The 2 mice killed on this day had gastric carcinoma. The main point of penetration of the tumors onto the serosa was at the pyloric ring. One carcinoma infiltrated scar tissue between the stomach and pancreas. Umbilicate lesions were seen in various stages of development, and neoplastic glands infiltrated the injection site and muscularis propria over a wide area. The tip ends of the tall cells lining some of the glands in the submucosa contained a vacuole filled with mucus, giving these cells the appearance of mucosal epithelial cells of the intestine. A second vacuole free of mucus was often interposed between the mucus-containing vacuole and the nucleus. No evidence of crystalline methylcholanthrene remained at the site of injection in either specimen. The stroma immediately surrounding the neoplastic tissue was infiltrated by plasma cells and

histiocytes and did not appear to be degenerated. Elsewhere in the stroma and in the walls of the telangiectatic vessels the fibrinoid change was less conspicuous in these 56-day specimens than in earlier specimens.

Days 70, 79, and 83.—Four specimens were examined. A carcinoma was found at 70 days and precarcinomatous lesions at 70, 79, and 83 days. In the 70-day specimen with the precancerous lesion, papillary-glandular and cystic structures confined to the submucosa and muscularis propria were in continuity with a deep mucosal crypt that opened onto the surface of the stomach. Scar tissue replaced much of the muscularis propria, and this may have limited the deeper extension of the neoplastic glands. In the 70-day specimen with gastric carcinoma, a fibrous and inflammatory ulcer had its base in the muscularis propria. Atypical spindle-shaped cells, suggestive of early sarcoma, were found in a fibrous serosal plaque adjacent to the carcinoma. There were also several enlarged nerve cells in a sympathetic ganglion near this plaque. Fibrinoid degeneration was absent in the stroma supporting the neoplastic glands and was minimal in the submucosa and telangiectatic blood vessels. Acicular spaces were absent from the inflammatory area in the submucosa.

Days 84, 91, and 98.—Of 9 specimens examined, 3 had precarcinomatous lesions (91, 91, and 98 days) and 6 had carcinoma (84, 84, 91, 98, 98, and 98 days; fig. 14). The precarcinomatous lesions were large and annular and almost completely encircled the distorted, tortuous, gastric antrum. One contained foci of squamous metaplasia. The carcinomas were composed of small, well-differentiated glands lined with a single layer of cells and of large, cystic, branched, atypical glands that infiltrated the muscularis propria at several separate points. Mitotic figures were normal in appearance. Two of the carcinomas were ulcerated, but in 4, any pre-existing defect in the mucosa was now occupied by neoplastic glands. In 1 animal the carcinoma had spread into perigastric adhesions, and in another there was a cluster of neoplastic glands within a blood vessel of the pancreas, indicating for the first time the spread of carcinoma beyond the confines of the stomach. The stroma around the neoplastic glands was infiltrated by plasma cells, lymphocytes, and granulocytes, but was free of fibrinoid change. Fibrinoid degeneration was present in the remnants of the original submucosa, in the walls of telangiectatic blood vessels, in the perivascular scars of the subserosa, in serosal plaques, in perigastric fat infiltrated by carcinoma, and in peritoneal adhesions. The reticulum supporting the neoplastic glands was healthy, while in fibrinoid areas it was degenerated.

Days 105 to 175.—Of 33 mice necropsied, 17 had precancerous lesions only, 12 carcinomas only (fig. 15), 1 adenoacanthoma and sarcoma, 1 adenocarcinoma and sarcoma, and 1 sarcoma. The neoplasms were in some cases plaquelike but not polypoid and extensively replaced the wall of the antrum. Extragastric spread of the cancers was a prominent feature. The sarcoma invaded both liver and pancreas, and, within the stomach, coexisted with a precarcinomatous lesion. Carcinoma infiltrated the gastric attachment of the mesentery in 2 cases, the gastrohepatic adhesions

in 3, the gastropancreatic adhesions in 1, and both the pancreas and the liver in 1. The mixed adenocarcinoma-sarcoma showed intermingling of both epithelial and connective tissue elements, and the tumor invaded an adhesion between the stomach and duodenum. The pattern of the elements composing the mixed adenoacanthoma-sarcoma that infiltrated the liver, pancreas, and perigastric adhesions was found to be as follows: The infiltrate in the adhesions was sarcomatous; the portion of the neoplasm confined to the stomach had a glandular pattern; all the extragastric portion of the carcinoma exhibited a mixed glandular and squamous pattern. This latter finding suggests that the squamous-cell element probably developed after this neoplasm had extended beyond the limits of the stomach. However, the proportion and distribution of the squamous elements in the precancerous lesions was not constant. Squamous elements might be confined to the surface of the growth, might be distributed evenly throughout it, or might be present only in the deepest portion. Squamous metaplasia was seen so much more frequently between 105 and 175 days than at earlier periods that it must be a relatively late manifestation in the neoplastic process. Serial sections revealed no connection between the metaplastic squamous epithelium of the mixed neoplasms and the squamous mucosa of the forestomach, indicating metaplasia of the glandular elements of the pyloric chamber. The neoplastic infiltrates in the submucosa and muscularis, which had been shown by serial sections to be separate and distinct from each other at earlier periods post injection, were now coalescent at the 105- to 175-day period and had lost their continuity with the surface glands. During this late stage of the experiment, small umbilicate lesions were observed side by side large carcinomas or extensive precancerous lesions, indicating the multicentric origin of cancer. It is probable that foci of cancer tissue may arise successively in point of time and become incorporated to form the final neoplastic lesion. Of two umbilicate lesions developing at the same given time, it is also possible that one may progress to carcinoma, while the other, more static, remains precancerous or may possibly regress or disappear.

The lesions of the blood vessels in all specimens examined in this latest period of the experiment were widespread and severe (fig. 16). They consisted of fibrinoid degeneration, telangiectasis, endarteritis, periarteritis, and small aneurysms. In one case there was thrombosis of a branch of the portal vein within the liver. Chronic inflammatory changes and scar tissue were prominent in association with most of the neoplastic lesions. There were many areas of fibrinoid degeneration that appeared bright red in the hematoxylin-eosin stain, purplish red in the periodic acid-Schiff reaction, and homogeneous and dark-colored with Wilder's silver; neither reticular nor collagenic fibers could be resolved in these degenerated areas.

Discussion

The present study emphasizes the complexity and evolutionary nature of the process of experimental carcinogenesis in the glandular stomach of

mice. The steps in the development of induced gastric carcinoma are essentially similar to those of induced carcinoma of the small intestine (*20*) and of the forestomach of mice (*21, 22*). The carcinomas of all three sites appear to develop from multicentric neoplastic foci and to originate from the deep mucosa in association with umbilicate lesions. The normal direction of differentiation and growth of the epithelium in the three sites is reversed under the influence of the methylcholanthrene. The reticular basement membrane of the original lamina propria degenerates and is succeeded by a new reticulum of normal appearance that forms around the neoplastic epithelium in the submucosa and persists in the tumors during their subsequent progression (*20–22*). Inflammation and degeneration of stroma and blood vessels, telangiectasis, endothelial proliferation, and thrombosis commonly accompany the development of these carcinomas. New umbilicate lesions continue to form in the superficial mucosa, while older umbilicate lesions show progressive neoplastic change during the entire process of carcinogenesis in the glandular stomach, the forestomach, and the small intestine. It may be that these umbilicate lesions are a manifestation of the toxic effects of the methylcholanthrene or the result of occlusive changes in the blood vessels.

Throughout the duration of this experiment the fibrinoid degeneration of the walls of blood vessels and of the stroma increased in extent, even after the methylcholanthrene had disappeared from the site of injection. Once initiated, this process of degeneration seemed to be irreversible, and either persisted or terminated in healing and scar formation. The early-developing gastric carcinomas, although confined to a small mucosal area in relation to the deposit of methylcholanthrene, involved so many otherwise intact glands that even the earliest stage of carcinoma observable could not possibly have derived from a single transformed cell. The preinvasive stage of experimental carcinoma was not associated with polyps, peptic ulcers, areas of chronic gastritis, intestinalization of the gastric mucosa (*15, 16, 23, 24*), or any lesion resembling the so-called "carcinoma *in situ*" of man (*25*). Intestinal metaplasia was observed in glands that had penetrated the submucosa. The few ulcers that did occur in the mouse's stomach seemed to have developed as erosions of umbilicate lesions, or as a sequela to occlusion of the blood vessels.

The study of serial sections of the glandular stomach of mice killed in close sequence afforded us the opportunity to observe the multicentric origin of cancer and to follow the infiltration of neoplastic glands into the wall of the stomach. In some instances this infiltration took place along the course of blood vessels at points of potential separation of the different coats of the viscus. The neoplastic glands usually penetrated the muscularis mucosae by way of a clean break through apparently healthy muscle tissue. Rarely was this coat pushed in advance of the infiltrating glands nor were strands of it carried along by the glands. The defect in the muscularis mucosae, narrow at first, later enlarged, and the edges of the muscle bordering it were not distorted in any way. The glands infiltrated the submucosa at several points lateral to, or above, the site of

injection of the methylcholanthrene, and the pyloric ring seemed to be a site particularly susceptible to glandular penetration. The neoplastic elements, once they reached the submucosa, proliferated to form congeries of anastomozing glands. Eventually, a conglomeration of all these neoplastic glands that had infiltrated the wall of the stomach in a steplike fashion constituted the total bulk of the carcinomatous tissue. In the stomach of the mouse with the first observed carcinoma (42 days post injection), neoplastic glands, still in continuity with their parent glands in the mucosa, infiltrated to varying depths the different coats of the stomach at the margin of the main mass of the tumor. This variation in tempo of the neoplastic process, as indicated by the varying depths to which neoplastic glands had penetrated, was observed in many other stomach specimens and indicated that, under the conditions of this experiment, cancer developed from multicentric foci.

The yield of carcinomas in the present experiment (table 1) was higher than the yield in an earlier experiment (table 2). The two experiments were similar with respect to the species and strain of animal, the preparation and the dose of 20-methylcholanthrene, and the diagnostic criteria for carcinoma. However, they differed in the number of mice used, in the proportion of the sexes, in the schedule for killing the mice, in the duration of the experiment, and in the technique for sectioning the stomach. In the present experiment all but 7 of 109 mice used were males; one to several animals were killed hourly, daily, biweekly, or weekly; the last mouse was necropsied 175 days post injection; the stomach specimens were serially sectioned. In the earlier experiment, there were 111 animals approximately equally divided as to sex (55 males and 56 females); all animals except those with palpable tumors and those that appeared ill were permitted to live out their lifespan; the last mouse was necropsied 662 days post injection; only a few sections were cut from each stomach specimen. In the present experiment, the first carcinoma was found in a mouse necropsied 42 days post injection. Of the 50 mice necropsied in the effective tumor period (42 to 175 days), 22 had adenocarcinoma, 1 a mixed adenocarcinoma and sarcoma, 1 a mixed adenoacanthoma and sarcoma, and 1 a sarcoma. In the earlier experiment (expt. 169) the first observed tumor, a mixed adenocarcinoma and sarcoma, was found in a mouse killed 90 days post injection. Of 106 mice necropsied in the effective tumor period (90 to 662 days), there were 2 adenocarcinomas, 1 adenoacanthoma, 5 mixed tumors consisting of adenocarcinoma and sarcoma, 1 mixed tumor consisting of adenoacanthoma and sarcoma, and 1 sarcoma. Thus, there were 3 tumors with a sarcomatous element in the first experiment and 7 in the second, an insignificant disparity in view of the greater duration of the first experiment. However, in the present experiment, 24 of 50 mice killed in the effective tumor period had a tumor with a carcinomatous element, whereas in the earlier experiment only 9 of 106 mice had such a tumor. This difference in the number of carcinomatous tumors could not be accounted for on the basis of the sex of the animals. It is possible that in the present experiment the serial

sections of the stomachs revealed some minute lesions of infiltrating carcinoma that would have been missed if only random sections of stomachs had been taken, as in the earlier experiment. However, this difference in technique could hardly account completely for the difference in the number of carcinomatous tumors. Had the mice in the present experiment lived as long as those in the earlier experiment, it might have been expected that most of their histologically evident carcinomas would have progressed and grown to be sizable tumors, and also that additional new histologically evident carcinomas would have developed. On the basis of this conjecture, more than 10 times the number of carcinomatous tumors should have developed in the earlier experiment. Since this did not happen, one must consider the possibility that, even though neoplastic glands invade the wall of the stomach and appear on the peritoneum, they not only fail to progress beyond this point but, instead, often regress.

The time of disappearance of the methylcholanthrene from the site of injection may be an important factor in the process of gastric carcinogenesis, and there is some support for this hypothesis from other studies. The initiation, progression, and growth of subcutaneous sarcomas induced by methylcholanthrene have been retarded by interruption of the contact between the susceptible tissues and the carcinogen during the latent period of carcinogenesis (*26*, *27*). In the present experiment no evidence of crystalline methylcholanthrene was found at the injection site in the stomach of any mouse killed 15 days post injection, and methylcholanthrene was absent in some specimens examined as early as 11 days. The methylcholanthrene may have disappeared from the injection site in the stomach as the result of destruction or transportation of the crystals by phagocytes, absorption by blood or lymph, or discharge of the hydrocarbon into the lumen of the stomach by way of one of the cryptlike lesions. With the thought in mind that the progression of tumor formation would be enhanced if a second dose of the carcinogen was introduced about the time the first dose had disappeared, we performed another experiment, in which we injected a second dose of methylcholanthrene into the precancerous gastric lesions of some mice and rats (*28*). This procedure resulted in a violent, necrotizing, inflammatory reaction attended by acute perforation of the stomach and by death. Although these results were disappointing, we still feel that some modification of this repeated dose technique might obviate the undesirable side effects and yet add the necessary stimulus to increase the yield of tumors; *e.g.*, modification of the dose of the carcinogen, selection of a less vulnerable site for injection, injection into the right gastroepiploic artery, a combination of injection of a carcinogen into the wall of the stomach with total-body irradiation or with the oral administration of a promoting agent or of the same or another carcinogen. The combination of such controlled variables with a standardized technique like that used in the two experiments cited in this paper might well give results that could be compared and evaluated more accurately than has been possible so far.

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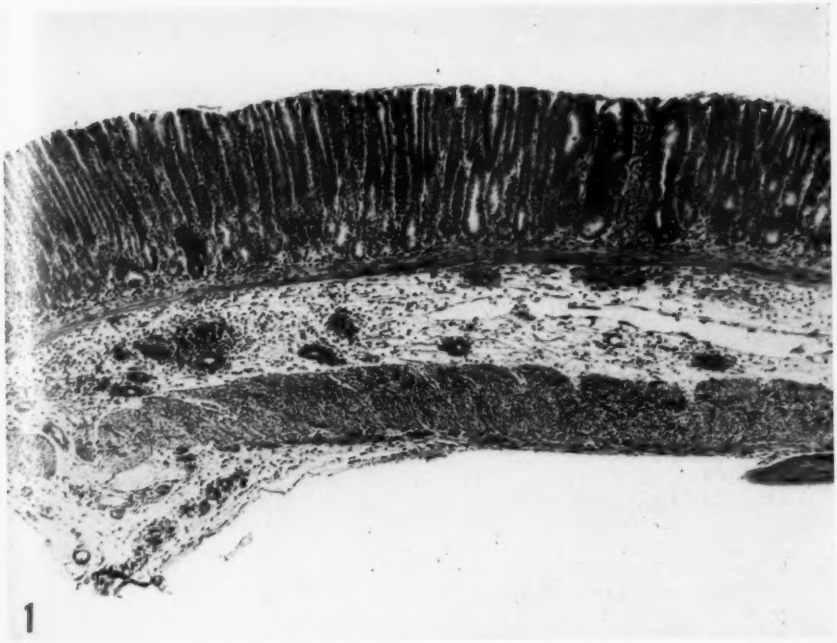
PLATE 99

FIGURE 1.—Glandular stomach of a mouse 8 days after injection of carcinogen, showing the thickness of the stomach wall at the periphery of the site of injection of methylcholanthrene. There are focal accumulations of granulocytes in the submucosa around the acicular spaces marking the location of the crystals of methylcholanthrene. Between and around the inflammatory foci the connective tissue of the submucosa is edematous, and there is a sparse infiltration of inflammatory cells. There are also inflammatory cells in the lamina propria at the base of the mucosa. The mucous membrane above the site of injection is atypical in that it shows dilatation of glands and proliferation and stratification of glandular cells, with change in polarity and elongation and budding of some of the basal glands. In adjacent sections colored by the periodic acid-Schiff reaction the mucus content of the mucosal cells overlying the inflammatory reaction in the submucosa has decreased, but the mucus is best preserved in the superficial third of the mucosa. Hematoxylin and eosin. × 78

FIGURE 2.—Glandular stomach of a mouse 8 days after injection of carcinogen. A high-powered view of an area of the submucosa shown in figure 1. A cuff of granulocytes surrounds acicular spaces from which crystals of methylcholanthrene have been dissolved. Between these inflammatory foci there are other inflammatory cells and fibroblasts. Hematoxylin and eosin. × 835



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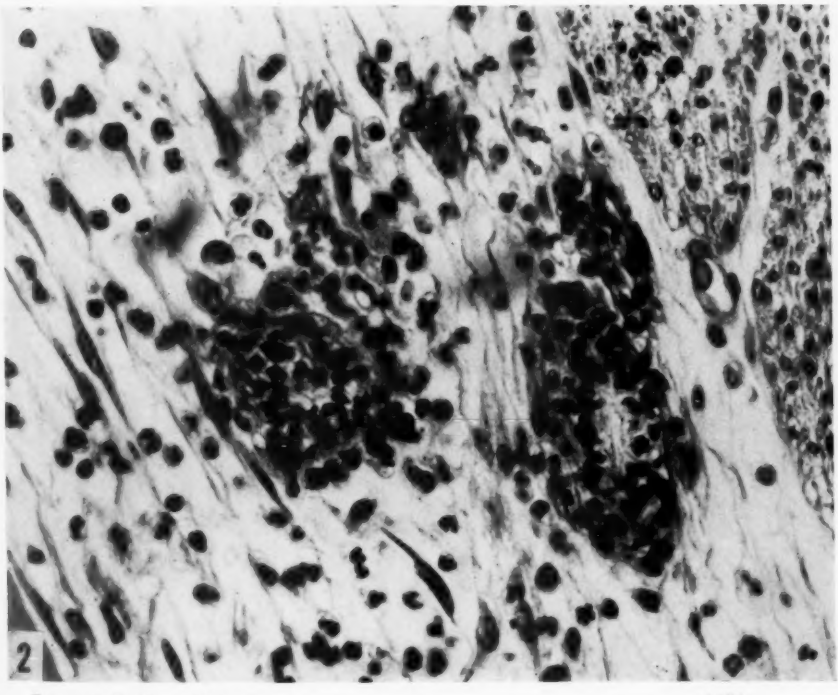

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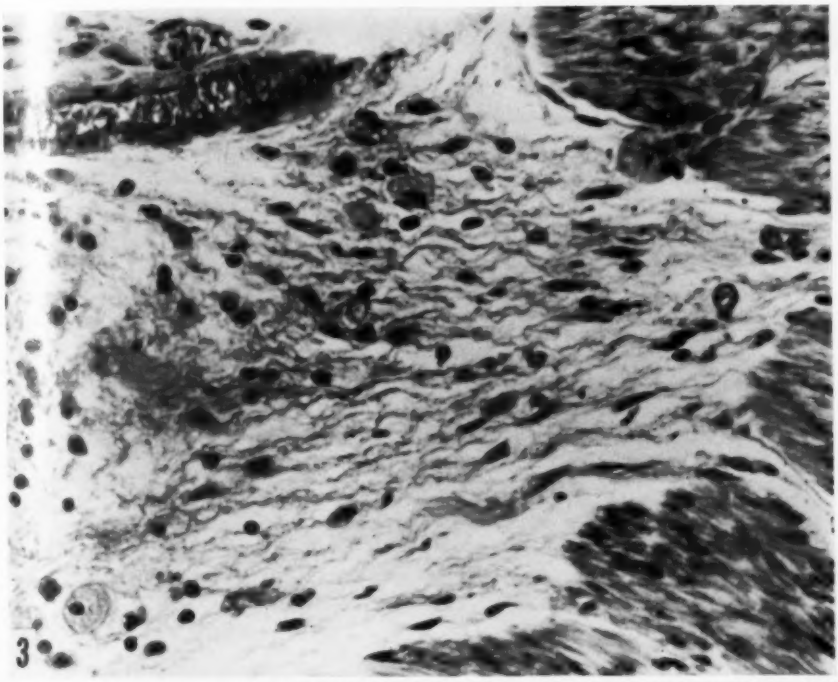
PLATE 100

FIGURE 3.—Glandular stomach of a mouse 8 days after injection of carcinogen. A high-powered view of an area of the submucosa from the specimen shown in figures 1 and 2 a little distance away from the most intense inflammatory cell infiltration. The collagen in the submucosa shows fibrinoid degeneration. There are a number of pyknotic nuclei and a few, large, swollen phagocytes. Hematoxylin and eosin. × 410

FIGURE 4.—Glandular stomach of a mouse 5 days after injection of carcinogen. There is a small, round, submucosal nodule which may represent early formation of sarcomatous tissue. This nodule is composed of spindle-shaped cells, either of fibroblastic or of smooth muscle origin. The nuclei are somewhat irregular, and some contain large nucleoli. Below the nodule the muscularis propria is thickened and somewhat fibrotic. In the overlying mucosa many of the cells lining the glands are hyperchromatic. Hematoxylin and eosin. × 265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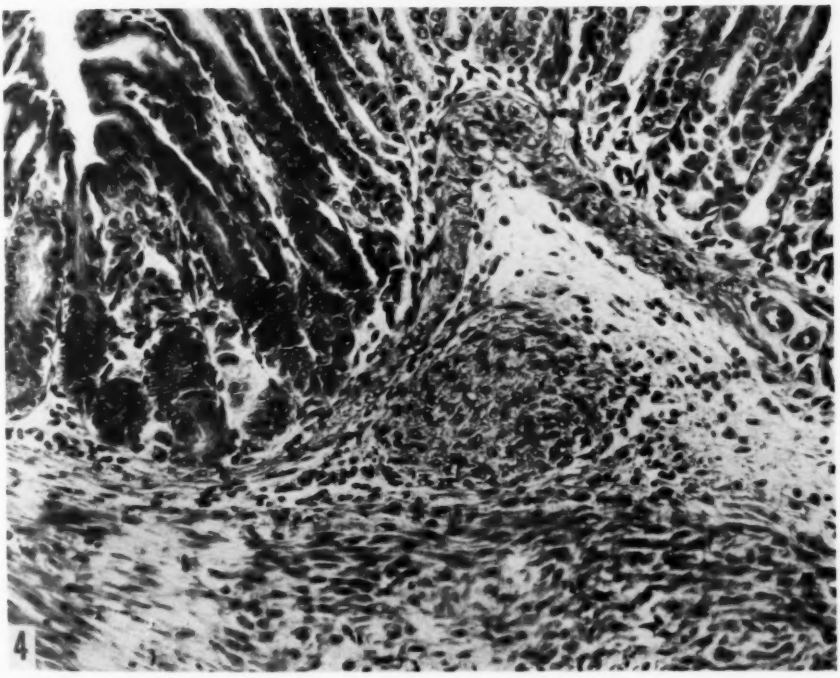
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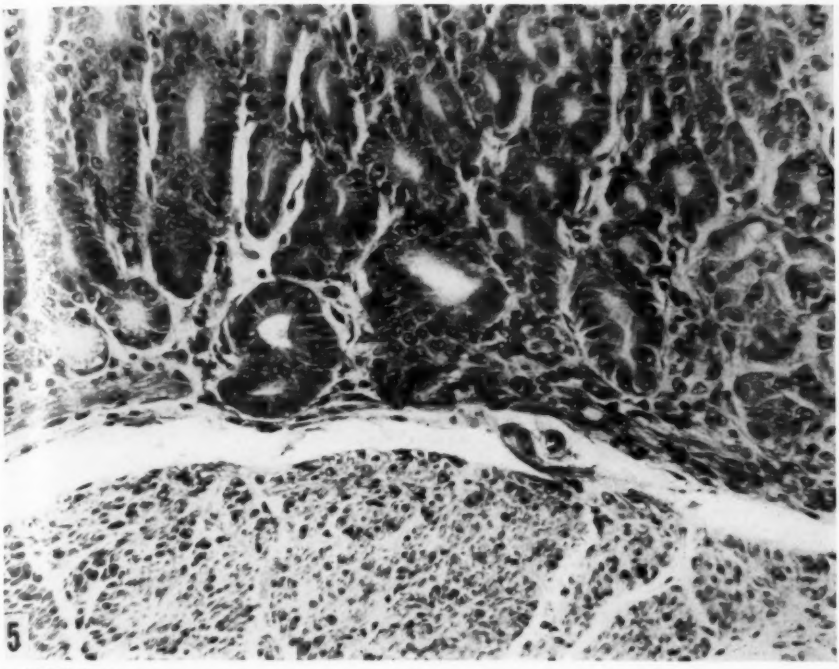
PLATE 101

FIGURE 5.—Glandular stomach of a mouse 5 days after injection of carcinogen, showing an area of the stomach lateral to the inflammatory infiltrate surrounding the injection site. The muscularis mucosae is infiltrated by basal glands at two separate points. Some of the mucosal glands are dilated and elongated and show stratification of the lining epithelial cells and changes in the polarity of their nuclei. There are a few, large, atypical, pale cells that contain brownish granules. In nearby areas several glands had infiltrated the submucosa, as revealed by serial sections. Hematoxylin and eosin. × 390

FIGURE 6.—Glandular stomach of a mouse 5 days after injection of carcinogen. In this section the atypical changes in the mucosa and the beginning of infiltration have occurred in a fold of the mucosa just proximal to the submucosal, inflammatory cell infiltrate visible on the *left*. There is a deep crypt lined by atypical glands, and beneath this the muscularis mucosae is attenuated; many of its fibers have disappeared and have been replaced by fibroblasts. The glands are dilated, irregular, and branched. Some of the cells that line the glands contain mucus, while others do not. At the apex of the crypt are large irregular cells that are illustrated in higher magnification in figure 9. Hematoxylin and eosin. × 100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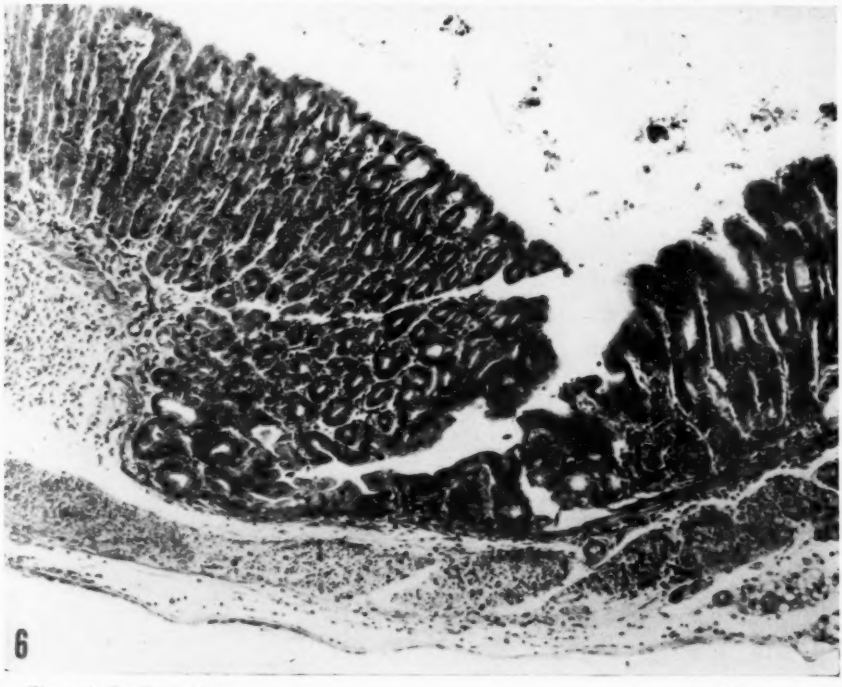
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PLATE 102

FIGURE 7.—Glandular stomach of a mouse 5 days after injection of carcinogen, showing the base of an umbilicate lesion bordered on either side by overhanging mucosa. A focus of glands has extended through the muscularis mucosae and into the submucosa. These glands surround a cryptlike depression that communicates with the lumen of the stomach. The glands bordering this crypt are composed of distorted epithelial cells. Many of these cells are columnar and, in the periodic acid-Schiff stain, contain fine granules. Other epithelial cells are spindle-shaped, as though subjected to stretching. Some acini are not intact and show reversal of the epithelial cells that point in the direction of the infiltration. Periodic acid-Schiff. × 125

FIGURE 8.—Glandular stomach of a mouse 9 days after injection of carcinogen. An atypical glandular structure lies within the base of the mucosa and the submucosa and has replaced the muscularis mucosae. The long axis of this glandular structure runs parallel to the long axis of the stomach. As revealed by the periodic acid-Schiff reaction, no stainable basement membrane could be found along the inferior border of this glandular structure where the basal cells are closely applied to the compressed stroma of the submucosa. These deep-lying cells are stratified, and their nuclei lie in different places. Some of them are arranged in the form of papillary excrescences. The cytoplasm of many of these cells is abundant, pale, and smooth. The left superior margin of this atypical glandular structure is composed of acini and solid nests of cells. This atypical submucosal gland is continuous with a nearby deep crypt that opens onto the surface of the mucosa at the base of an umbilicate lesion. Hematoxylin and eosin. × 125

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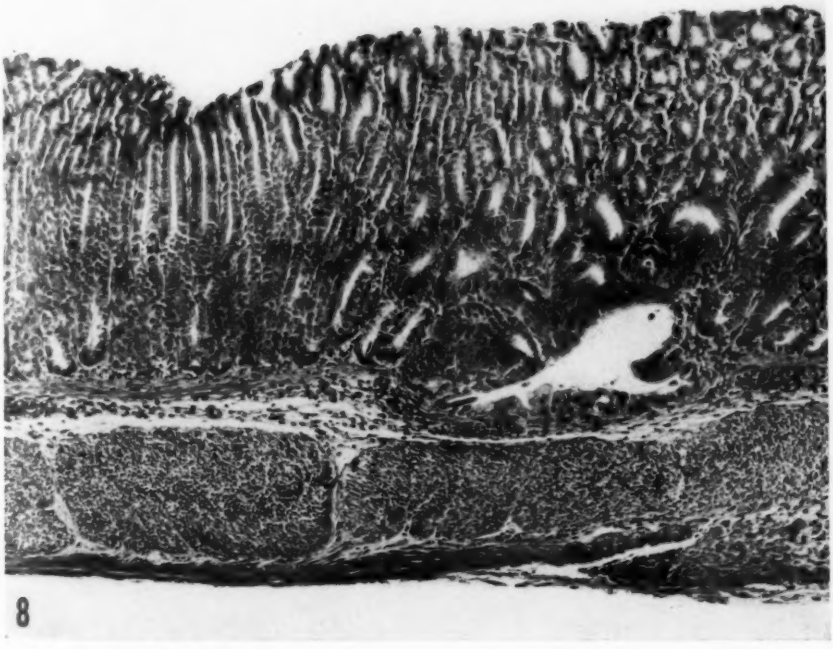

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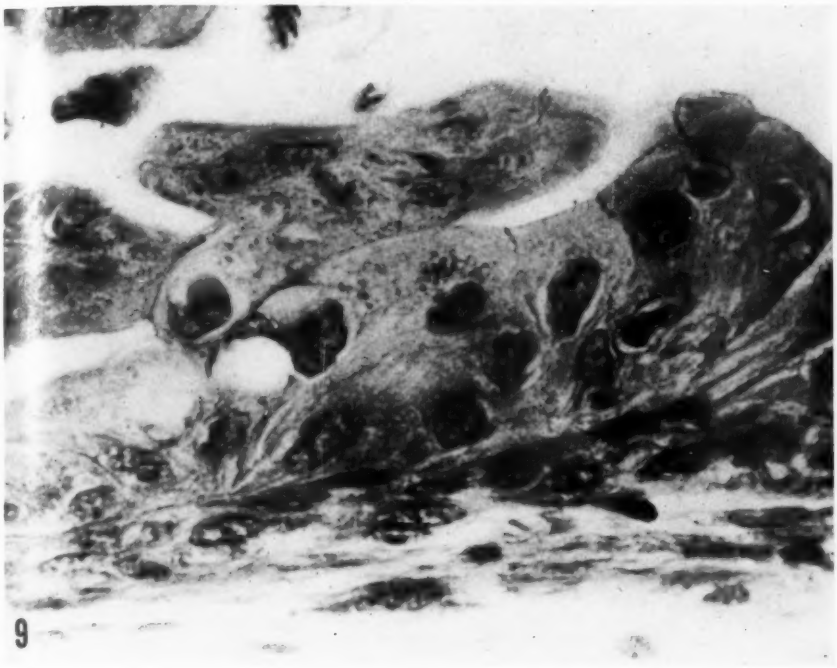
PLATE 103

FIGURE 9.—Glandular stomach of a mouse 5 days after injection of carcinogen. This is a high-powered view of the apex of the crypt shown in figure 6. The cells are slightly piled up and pseudostratified; many of the cells in this field are large and somewhat resemble squamous cells. The nuclei vary in size, morphology, polarity, and staining characteristics. The cytoplasm is abundant, frequently vacuolated, and may contain clusters of fine, brownish granules. Hematoxylin and eosin. × 1,180

FIGURE 10.—Glandular stomach of a mouse 11 days after injection of carcinogen, showing an area of atrophic mucosa constituting a part of an umbilicate lesion, the margins of which are not shown in this illustration. There is atypicality of the cells and glands of the mucosa and a minimal inflammatory cell infiltration in the wedge of submucosa to the *left*. In the *center* of the field a complicated, dilated, branched gland has impinged upon the muscularis mucosae and has displaced it downward. The base of this gland is flattened, the lining is everywhere intact, and the cells are hyperchromatic, enlarged, and pseudostratified. Other sections through this block reveal glands that have infiltrated the submucosa. Hematoxylin and eosin. × 240



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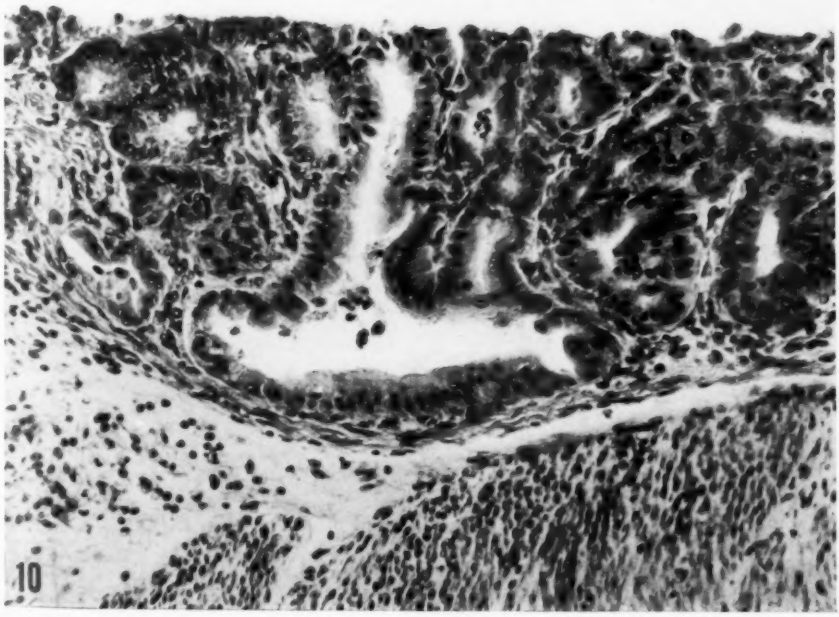

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PLATE 104

FIGURE 11.—Glandular stomach of a mouse 11 days after injection of carcinogen. A single gland has penetrated the muscularis mucosae, and its deepest aspect lies within the submucosa. This gland has apparently gone directly through the muscularis mucosae without distorting it. The deepest portion of the gland shows flattening of the epithelial cells and degeneration and desquamation of some of them. Most of the rest of this gland is lined by hyperchromatic, somewhat atypical, epithelial cells. There is an inflammatory reaction in the submucosa. A study of the complete serial sections of this specimen of stomach revealed several other similar lesions in which single glands had infiltrated the submucosa. Hematoxylin and eosin. × 420

FIGURE 12.—Glandular stomach of a mouse 11 days after injection of carcinogen, showing a midplane section through a submucosal neoplastic lesion that is continuous with a cryptlike mucosal lesion that opens into the lumen of the stomach a few sections beyond the plane of this section. The submucosa has increased in width as the result of edema and inflammatory cell infiltrate. Within the connective tissue of the submucosa there are acicular spaces, and some of the fibrillar material has degenerated. There is expansion of the glandular lesion in the submucosa. The glands composing the neoplastic nodule are branched, and many of them show a thin layer of mucus on the surface cells. The area of penetration through the muscularis mucosae appears to be sharply delineated. There are many more mitotic figures in the cells of the midmucosa than in the cells of the submucosal infiltrate. Periodic acid-Schiff. × 60

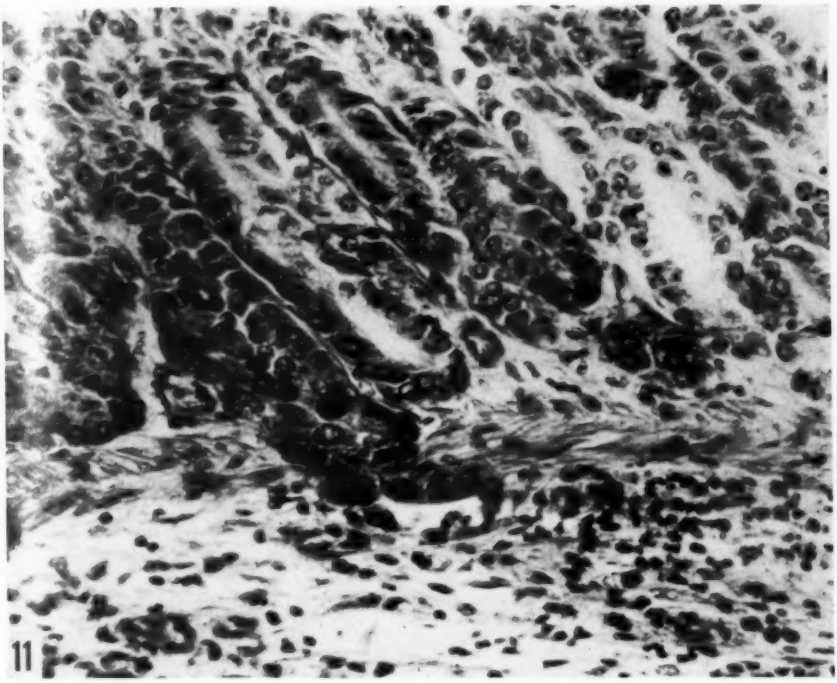

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PLATE 105

FIGURE 13.—Glandular stomach of a mouse 15 days after injection of carcinogen. A cryptlike defect extends through the mucosa into the submucosa and opens into the lumen of the stomach. The portion of the lesion in the submucosa has expanded. The defect is bordered by atypical glands and cells, and there is evidence of degeneration and desquamation. Complete serial sections of this portion of the glandular stomach revealed the presence of several separate and distinct cryptlike lesions similar to those shown in the illustration. In the glandular mucosa bordering this lesion the glands are irregular and atypical. Hematoxylin and eosin. × 82

FIGURE 14.—Glandular stomach of a mouse 98 days after injection of carcinogen. A coronal section through an obstructive lesion of the antrum showing the dilated stomach above and the duodenum below. *Grossly*, there is a napkin-ring deformity of the antrum and marked thickening of the mucosa. *Microscopically*, the mucosa on both sides of the antrum is converted into neoplastic nodules. These nodules occupy the submucosa of the section on the *right*. On the *left*, the neoplastic tissue extends into fibrous inflammatory tissue on the serosa. Hematoxylin and eosin. × 22

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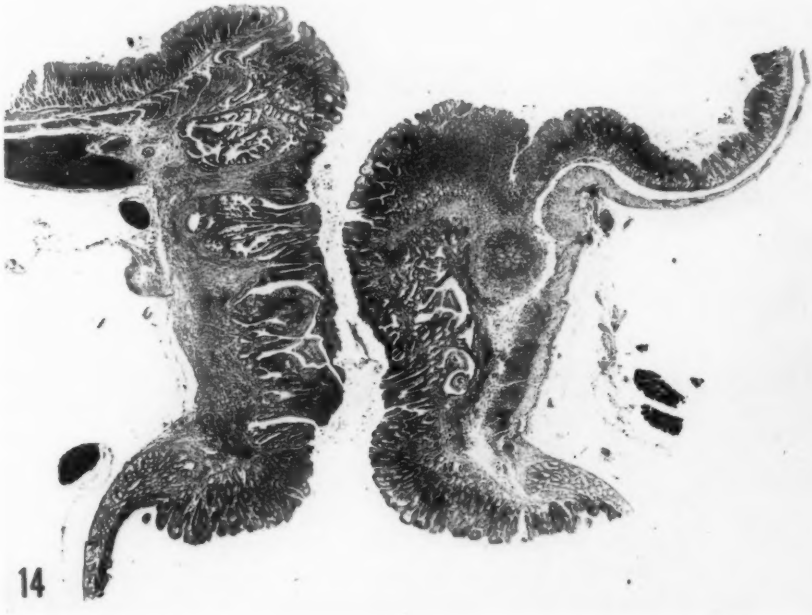

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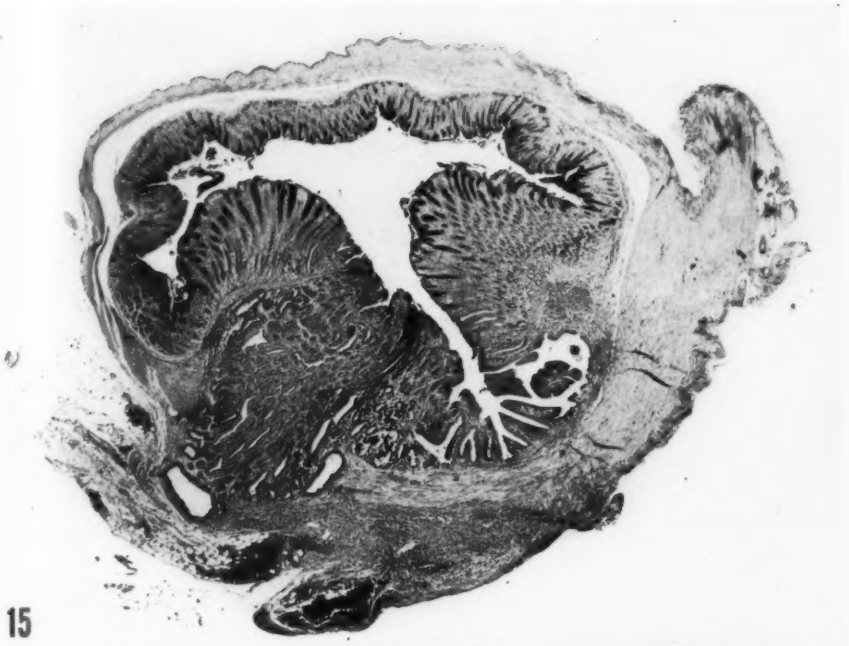
PLATE 106

FIGURE 15.—Adenocarcinoma of the glandular stomach of a mouse 120 days after injection of carcinogen. The neoplastic glandular lesion has infiltrated all coats of the wall of the stomach and has extended into the surrounding fibrofatty tissue and into a fibrous inflammatory plaque on the peritoneum. A crypt that communicates with the lumen of the stomach extends into the deep portion of the neoplastic nodule. Periodic acid-Schiff. × 20

FIGURE 16.—Glandular stomach of a mouse 175 days after injection of carcinogen, showing the base of a diverticular lesion that is bound to the liver by a fibrous inflammatory adhesion. At the *upper left* of the illustration remnants of the lining epithelium are present, and these epithelial cells are continuous with a nearby large precancerous nodule, not shown in this illustration. The apex of the diverticular lesion is bordered by degenerated collagen that is deeply acidophilic. At the *lower right* margin of the illustration there is a section of an artery that shows fibrinoid degeneration of its wall. Serial sections revealed that at deeper levels the wall of this vessel was almost totally destroyed and deeply acidophilic; probably the deep cryptlike ulcer was secondary to interference with the blood supply due to damage to the wall of the nutrient vessel. Periodic acid-Schiff. × 150



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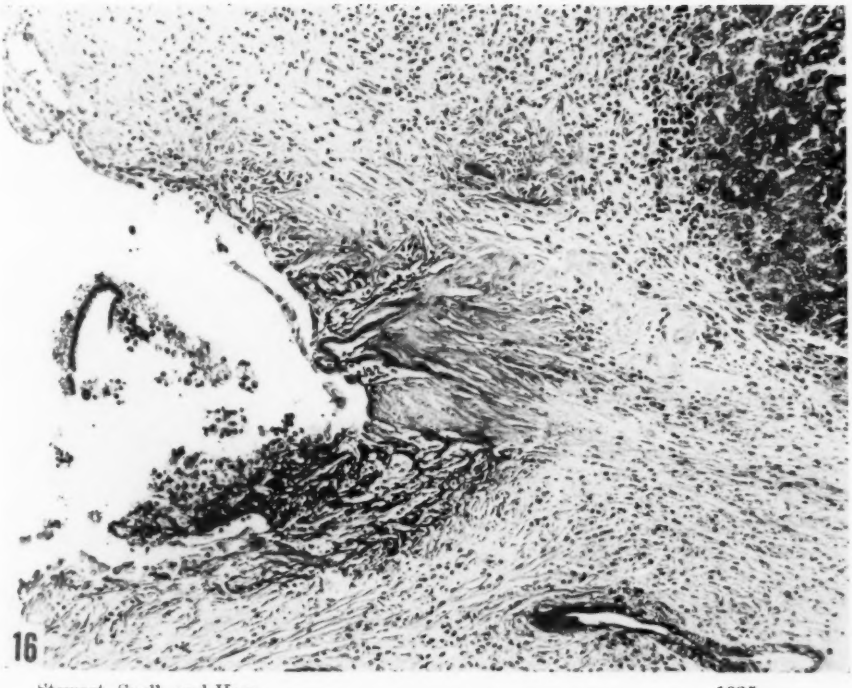

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Announcements

Summer Programs for College and Secondary School Students

Roscoe B. Jackson Memorial Laboratory
Bar Harbor, Maine

The Roscoe B. Jackson Memorial Laboratory offers college and secondary school students who are interested in possible careers in biology, medicine, or psychology a unique opportunity to gain experience in actual research work.

Aim of the Program

1. To find and encourage scientific ability at an early age.
2. To bring talented students in direct contact with research workers and their materials.
3. To stimulate in students the desire for knowledge and the discovery of new knowledge.
4. To help students select careers for which their interests and aptitudes fit them.

Course of Study

1. Daily lectures in various biologic subjects, such as genetics, physiology, pyschology, and cancer, given by members of the Laboratory staff and invited guest lecturers. The lectures are followed by laboratory and research periods.
2. The students work on research projects, singly or in small teams, under the sponsorship and guidance of staff members.
3. Reports on the research projects in a form similar to a scientific paper are prepared by each student. There are no formal examinations.

Student Expenses

Tuition is free. A fee of $200 for secondary school students and $150 for college students is charged for board and room for the 10-week period. Scholarships to cover the cost of board and room are available.

Application blanks and additional information may be obtained by writing to:

Dr. William S. Murray, Associate Director
Roscoe B. Jackson Memorial Laboratory
Bar Harbor, Maine

Precollegiate Summer Science Program

The Worcester Foundation for Experimental Biology affords interested high school juniors and seniors an opportunity to learn and study advanced scientific techniques in biology and biochemistry. Further details may be obtained by writing to:

Mr. Frederick R. Avis
Chairman, Science Department
Saint Mark's School
Southborough, Massachusetts

ERRATUM

Volume 21, Number 2, August 1958

Page 286, footnote 8: In the paper entitled "Effects of Donor and Host Lymphoid and Myeloid Tissue Injections in Lethally X-Irradiated Mice Treated with Rat Bone Marrow," by Santos and Cole, *for* "No lysed or lyophilized control was carried out for rat thymus, rat spleen homogenate, lysate, and lyophilizate. All rat thymus homogenate contained bacteria, but no quantitative differences in these preparations were noted when plate cultures were made" *read* "No lysed or lyophilized control was carried out for rat thymus. Rat spleen homogenate, lysate, lyophilizate, and rat thymus homogenate all contained bacteria, but no quantitative differences in these preparations were noted when plate cultures were made."